VP1-MEDIATED REPRESSION OF ALPHA-AMYLASE GENES IN DEVELOPING MAIZE ALEURONE

BY

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The Viviparous-1 (VP1) transcriptional activator of maize is required for abscisic acid-induction of maturation-specific genes late in seed development. In the presented work, it is shown that, in addition, VP1 inhibits precocious induction of the germination-specific α-amylase genes in aleurone cells of the developing seed. In developing seeds of the somatically unstable *vp1-m2* mutant, hydrolase activity was de-repressed specifically in endosperm sectors underlying *vp1* mutant aleurone. Moreover, in transient expression experiments based on particle bombardment of aleurone tissue, a barley high-pI α-amylase promoter-GUS fusion construct (Amy-GUS) was induced in developing *vp1* mutant aleurone cells but not in wild-type aleurone cells. A direct role of VP1 in repression of Amy-GUS is suggested from the finding that co-expression of recombinant VP1 in *vp1* mutant aleurone cells strongly inhibited expression of Amy-GUS. Hence, VP1 expression in the developing seed appears to integrate the control of two developmental programs, seed maturation and seed germination.

Over-expression of VP1 also inhibited Amy-GUS expression in aleurones of wild-type germinating maize and barley seeds. In barley aleurone cells, VP1 specifically repressed induction of Amy-GUS by gibberellic acid (GA), while in maize aleurone tissue, VP1 inhibited a GA-dependent as well

as an apparent GA-independent activity. Deletion of the acidic transcriptional activation domain of VP1 did not affect the inhibitory activity, indicating that VP1 has a discrete repressor function. Further deletion analysis of VP1 showed that domains essential for repression of Amy-GUS are distinguishable from domains required for activation of the maturation-related genes *Em* and *C1*.

The role of the embryo in the expression of Amy-GUS in developing maize aleurone cells was studied. Amy-GUS was de-repressed in *vp1* mutant aleurone in seeds that either carried a viviparous embryo or aborted the embryo early in development but not in seeds with a normal, non-viviparous embryo. This suggests that a normal embryo contributes a diffusible signal with inhibitory effect on Amy-GUS expression in the aleurone. Amy-GUS was partially de-repressed in wild-type aleurone cells of embryo-less seeds, suggesting that both *Vp1* expression in the aleurone and a non-viviparous embryo are required for complete repression of α-amylase genes in the developing maize aleurone.

INTRODUCTION

The formation of seeds is a unique characteristic of higher plants which promotes dispersal of the species and allows interruption of the life cycle during unfavorable environmental conditions. To survive in the dehydrated state, plant embryos undergo an adaptation process during late stages of seed formation (maturation phase) which renders them tolerant to desiccation and gradually causes arrested growth. In maize and other cereals, the outermost layer of the seed endosperm (aleurone layer) also undergoes a maturation process and remains viable through desiccation.

Seed maturation is associated with the activation of a variety of genes encoding storage proteins and various hydrophilic, late-embryogenesis-abundant (LEA) proteins which possibly function as desiccation protectants (Dure et al. 1989; Skriver and Mundy 1990). Analysis of viviparous mutants in maize has demonstrated that the developmental program of seed maturation is controlled by at least two factors, the hormone abscisic acid (ABA) and the product of the *Viviparous-1* (*Vp1*) gene (Robertson 1955; Neill et al. 1986). Developing *vp1* mutant embryos are distinct from ABA-deficient embryos in that they exhibit a reduced sensitivity to ABA in culture (Robichaud et al., 1980; Robichaud and Sussex, 1986). In addition to causing vivipary, the *vp1* mutation blocks synthesis of anthocyanins in embryo and aleurone tissues (Robertson, 1955; Dooner, 1985). The *vp1* mutant phenotype is restricted to seed tissues. Mutant embryos rescued prior to desiccation develop into apparently normal, fully fertile plants with normal patterns of anthocyanin accumulation.

The *Vp1* gene was cloned by transposon tagging (McCarty et al., 1989a). It encodes a 2500-nucleotide mRNA that is expressed specifically in embryo and endosperm tissues of the developing seed. Within the endosperm, *Vp1* expression may be limited to the aleurone layer because so far no mutant phenotype has been detected in the starchy endosperm. This is

consistent with the relatively low abundance of *Vp1* message in whole endosperm extracts (McCarty et al., 1989a).

Vp1 encodes a novel, 73 kD protein with a functional acidic transcriptional activation domain (McCarty et al., 1991). Over-expression of VP1 in maize protoplasts *trans*-activated reporter constructs containing late-embryogenesis-specific promoters: *C1*, a maize gene that encodes a transcription factor required for anthocyanin synthesis in the seed, and *Em*, a wheat *LEA* gene (Hattori et al., 1992; McCarty et al., 1991). In agreement with the phenotype of ABA-deficient mutants, VP1-activation of *Em* was strongly dependent on the presence of exogenous ABA (McCarty et al., 1991). These functional data confirm that VP1 plays a central role in the induction of seed maturation.

Following imbibition of mature non-dormant seeds, expression of maturation-specific genes is terminated and expression of a new set of genes related to the developmental program of seed germination is executed (Comai and Harada, 1990). In rehydrated cereal seeds, the germination-specific α-amylase genes which encode starch-hydrolyzing enzymes are induced in the aleurone cells by the hormone gibberellic acid (GA) that is secreted by the embryo early in germination (Jacobsen and Chandler, 1987). They are constitutively expressed in de-germed seeds of the barley GA-response mutant *slender* (Chandler, 1988; Lanahan and Ho, 1988), and their induction can be antagonistically inhibited by application of ABA (Jacobsen and Chandler, 1987).

Expression of the normally consecutive programs of seed maturation and seed germination is under strict developmental control. Precocious induction of germination-related events prior to seed maturity appears to be actively repressed. In developing seeds of cereals and maize, no α-amylase activities are found prior to seed maturity (Evans et al., 1975; Nicholls, 1979; Comford et al., 1986; Garcia-Maya et al., 1990; Oishi and Bewley, 1990). Moreover, α-amylase genes are unresponsive to applied GA (Nicholls, 1979; Comford et al., 1986; Garcia-

Maya et al., 1990; Oishi and Bewley, 1990). It has been suggested that the presence of ABA in developing seeds is responsible for the inhibition of α-amylase genes at this developmental stage (King, 1976). However, treatment of maize developing seeds with the ABA synthesis inhibitor flouridone was not sufficient to sensitize the aleurone cells to GA, suggesting the action of additional factors in repressing α-amylase genes in the developing seed (Oishi and Bewley, 1990).

The objective of this study was to elucidate a role of VP1 in the negative regulation of α-amylase gene expression in the developing maize seed. It is demonstrated that VP1 – in addition to activating seed maturation programs – blocks precocious induction of germination-specific α-amylase genes in the developing maize seed. A somatically unstable *vp1* mutant is described that displays de-repression of hydrolase activity specifically in *vp1* mutant sectors of the aleurone. Using a transient expression approach, it is shown that expression of recombinant VP1 in aleurone cells of maize and barley strongly inhibits expression of an α-amylase promoter-GUS reporter gene (Amy-GUS). Evidence is provided indicating that VP1 specifically represses GA-induction of Amy-GUS in aleurone of germinating barley seeds. It is also shown that deletion of the acidic activation sequence of VP1 does not affect VP1 repressor activity, indicating that VP1 has a discrete repressor function. Thus, it is suggested that the coupled activator and repressor functions of VP1 play a key role in integrating the control of the normally not simultaneously occurring maturation and germination programs in the seed.

REVIEW OF LITERATURE

Developmental and Hormonal Regulation of Seed Maturation

The biochemical mechanisms allowing seed tissues to tolerate extreme desiccation remain unclear. Many studies have implicated soluble sugars in desiccation protection. One of the suggested functions of soluble sugars is the protection of membranes which are often considered a primary site of desiccation damage (Crowe et al., 1992). It is thought that hydroxyl constituents of sugars substitute for water during dehydration and thereby stabilize membrane structures in the dehydrated state (Crowe et al., 1992). In agreement with this, di- and oligo-saccharides, especially sucrose and in some species raffinose and stachyose, increase in concentration in maturing seeds (Amuti and Pollard, 1977) and in desiccating pollen grains (Hoekstra et al., 1989). Their accumulation has been correlated with the acquisition of desiccation tolerance (Hoekstra and van Roekel, 1988; Koster and Leopold, 1988; Chen and Burris, 1990; Leprince et al., 1990; Blackman et al., 1992; Crowe et al., 1992). However, recent comparative studies using desiccation intolerant mutants or recalcitrant (desiccation intolerant) species found no positive correlation between oligo-saccharide content in the seed and the development of desiccation tolerance (Ooms et al., 1993; Still et al., 1994). Data of one of the studies suggest that a low ratio of mono- to oligo-saccharides may be the critical factor rather than the absolute amount of soluble sugars (Ooms et al., 1993) This suggests that sucrose may be involved in the formation of "glass" during dehydration which is promoted by oligosaccharides and inhibited by monosaccharides (Ooms et al., 1993). A "glass" is a liquid of high viscosity, such that it stops or slows down all chemical reactions requiring molecular diffusion, and thus, might conserve tissue structures during dehydration (Bruni and Leopold, 1991).

A second characteristic that may be involved in rendering the seed tolerant to desiccation is the synthesis and accumulation of specific proteins late in seed development (late-embryogenesis-abundant proteins, LEAs). The direct function of LEAs is unknown but based on their high degree of hydrophilicity (Dure et al., 1989) they are assumed to stabilize the structure of cellular proteins during dehydration (Skriver and Mundy, 1990; Dure, 1993).

Equally uncertain remain the mechanisms that arrest embryo growth and prevent precocious germination prior to seed maturity. Because immature embryos excised from the seed and placed in culture are capable of germinating readily, precocious germination of the embryo in vivo may be actively suppressed by a process that is dependent on an intact seed. Evidence from embryo culture experiments has implicated two factors in suppression of precocious germination: restricted water uptake (low water potential in the seed) and the hormone abscisic acid (ABA). Both factors, when imposed on cultured embryos, inhibited germination (reviewed in Quatrano, 1987; Kermode, 1990). Indeed, the osmotic potential of developing soybean embryos has been shown to be even more negative than that of the osmoticum used to inhibit germination of isolated embryos (Xu et al., 1990). Similarly, ABA concentrations increase early in the seed maturation phase (Quatrano, 1987) and may thus play a role in arresting embryo growth.

In summary, the physiological processes responsible for acquisition of desiccation tolerance and arrest in embryo growth are poorly understood. In the past 15 years, the focus of research has shifted to the identification of regulatory factors that control the activities of late embryogeny, especially at the level of gene expression. From its discovery in the 1950s, ABA has been a factor of interest because a rise in seed ABA concentration correlates well with the onset of maturation events. Normally, ABA concentrations peak at the time of maximum dry weight accumulation in the seed and then decrease to low concentrations towards seed maturity (Quatrano, 1987). A function of ABA in initiating maturation events and suppressing precocious germination of the developing embryo was confirmed by analyses of mutants defective in late embryogeny and studies of gene expression.

Isolation of Mutants Affected in Seed Maturation

Genetic deficiencies in seed maturation manifest themselves in precocious germination (vivipary) or reduced dormancy. Severely affected mutants exhibit additional features, such as intolerance to desiccation and reduced accumulation of seed storage proteins and LEAs.

Mutants have been most intensively isolated and analyzed in maize and *Arabidopsis*. Maize has been a model species for studying genetics for many years, mostly because its monoecious flower structure in combination with self fertility has allowed easy outcrossing and selfing. Moreover, the identification of several maize transposable elements (Ac/Ds, Spm/En, Mu) has made it possible to generate transposable element-induced mutants and subsequently clone the mutated locus using the transposon as a tag. *Arabidopsis* has become a powerful model species for a variety of reasons (such as short generation time, small genome size (Meyerowitz, 1987; Meyerowitz, 1994). Rather recently, cloning of mutated genes has been achieved by *Agrobacterium* transformation-mediated T-DNA tagging or chromosome walking (positional cloning).

Many maize viviparous mutants that arose spontaneously were collected and described already during the first half of this century (Eyster, 1931; Mangelsdorf, 1930; Robertson, 1955). Transposon-tagged mutants were induced more recently (e.g. McCarty et al., 1989a,b). In *Arabidopsis*, many mutants displaying vivipary or reduced dormancy have been isolated by screening chemically mutagenized seed for germination on medium containing ABA at a concentration that inhibits germination of wild-type seeds. Other mutants have been isolated by screening for the ability to germinate in the absence of GA which is normally required. Here, GA contents in the seed were reduced either by treatment with a GA-synthesis inhibitor or by using a mutant deficient in GA-biosynthesis. Recently, screening of transgenic lines produced by *Agrobacterium*-mediated seed transformation (Feldmann, 1991) for mutants defective in late embryogeny has revealed additional loci that control induction of seed maturation/suppression of precocious germination. Taken together, the identified mutants fall into three classes: 1) ABA-

deficient mutants, 2) ABA-insensitive mutants and 3) mutants affected in a thus far unknown mechanism.

ABA deficient mutants

Five ABA-deficient mutants have been identified in maize (*vp2*, *vp5*, *vp7*, *vp8*, *vp9*, Neill et al., 1986). Four of these mutants (*vp2*, *vp5*, *vp7*, *vp9*) lack carotenoids in addition to ABA (Robertson, 1955). These mutants accumulate various intermediates of the carotenoid biosynthesis pathway, indicating that they have lesions in carotenoid biosynthetic enzymes (Robertson et al., 1978). The deficiency in both carotenoids and ABA confirms that carotenoids are the precursor for ABA-biosynthesis in plants. Phenotypically, these mutants are viviparous, display a pale yellow to white coloring of the normally orange-yellow endosperm and form a lethal, white seedling which is deficient in chlorophyll due to photobleaching caused by the lack of carotenoids (Robertson, 1955; Anderson and Robertson, 1960).

Only one vivlparous mutant (*vp8*) is deficient in ABA without affecting carotenoid synthesis. *vp8* mutant seedlings are viable but form plants of severely dwarfed stature (Robertson, 1955). The biochemical lesion of this mutant is unknown. It may be deficient in a later step in the ABA-biosynthesis pathway that is involved in the conversion of the carotenoid xanthophyll to ABA (Zeevaart and Creelman, 1988). Whether the lack of ABA causes dwarfism has not been examined thus far.

Vivipary in maize is determined by the genotype of the embryo and is entirely independent of the genotypes of the mother plant or the endosperm. Viviparous seeds segregate at the expected ratio on a heterozygous mother plant, indicating that ABA contributed by the mother plant - mainly early in seed development - does not play a role in preventing vivipary. The relative contribution of embryo and endosperm in preventing vivipary can be assessed by the use of TB-translocations which make it possible to generate seeds with embryo and endosperm of different genetic constitution (e.g. a seed with a *vp5* mutant embryo and a wild-type endosperm, or vice versa; Roman, 1947; Beckett, 1993). In these experiments, seeds with a *vp5* mutant embryo and a wild-type endosperm were viviparous, while seeds with a wild-

type embryo and a *vp5* mutant endosperm were not (Robertson, 1952). Similar results were obtained with other ABA-deficient mutants (Robertson, 1955). Thus, vivipary in these mutants is entirely conditioned by the lack of ABA production in the embryo. The endosperm does not play an active role in preventing vivipary of a genetically viviparous embryo. This is consistent with later findings showing that the embryo is the major source of ABA produced in the developing seed (Zeevaart and Creelman, 1988).

In *Arabidopsis*, only one ABA-deficient mutant (*aba*) has been identified. It was isolated in a genetic screen selecting for the ability to germinate of the normally non-germinating GA-deficient *ga-1* mutant (Koorneef et al., 1982). The *aba* mutant is impaired in the epoxidation of the carotenoid zeaxanthin (Duckham et al., 1991) and thus displays normal accumulation of carotenoids. No carotenoid deficient mutants have been isolated in any genetic screen, which may reflect the predicted lethality of such mutations. The *aba* mutant produces plants that show increased withering of stems, leaves and siliques and an enhanced rate of water loss which is probably caused by reduced stomata closure upon water stress (Koorneef et al., 1982). *aba* mutant seeds exhibit strongly diminished seed dormancy. Wild-type *Arabidopsis* seeds normally require cold and light treatments to break imposed seed dormancy and allow germination to occur, whereas a high percentage of *aba* mutant seeds germinated readily without a need for dormancy-breaking treatments (Koorneef et al., 1982). However, in contrast to ABA-deficient mutants in maize, even severe *aba* alleles that reduce ABA levels in the seed below the level of detection produce seeds that are non-viviparous and desiccation tolerant (Koorneef et al., 1982). Hence, ABA may not be required for the induction of desiccation tolerance in *Arabidopsis*. However, leakiness of the *aba* mutant cannot be ruled out. The nature of the performed mutant screen which selected for the ability of mature, dry seeds to germinate may not allow the identification of more severely affected mutants. Possibly, residual, very low concentrations of ABA that may be present in *aba* mutant seed are sufficient to prevent vivipary (Koorneef et al., 1989). To test this, a mutant screen could be performed that selects for the ability of seeds to

germinate precociously late in seed development. Such a screen has proven successful in isolating *abi3* and other mutants (Keith et al., 1994).

Wild-type developing seeds of *Arabidopsis* accumulate ABA as a dual peak, an earlier maternally-derived one and a later embryo-derived one (Karssen et al., 1983). Reciprocal crosses between wild-type and *aba* mutant plants demonstrated normal dormancy in the absence of maternal ABA but not in the absence of embryonic ABA. Moreover, induction of dormancy, as judged from the acquired inability of the developing seed to germinate precociously, correlated well with the later peak of ABA accumulation (Karssen et al., 1983). Hence, acquisition of a dormant state is dependent on ABA produced by the embryo and is normally independent of ABA provided by the mother plant.

ABA-insensitive mutants

ABA-insensitive mutants of maize and *Arabidopsis* accumulate normal or higher concentrations of ABA in developing seeds as compared to wild-type (Neill et al., 1986; 1987; Koornneef et al., 1984). However, while the mutant phenotype of ABA-deficient mutants can be complemented by exogenous application of ABA, ABA-insensitive mutants continue to display vivipary or reduced dormancy in the presence of added ABA (Raubichaud et al., 1980; Raubichaud and Sussex, 1986; Koornneef et al., 1984). In a maize mutant, it was shown that the reduced sensitivity to ABA was not caused by a deficiency in ABA transport or metabolism (Raubichaud and Sussex, 1986). Thus, ABA-insensitive mutants are likely to be affected in ABA signal transduction.

A single locus (*Vp1*) regulating ABA-sensitivity has been identified in maize. *vp1* mutant embryos do not acquire desiccation tolerance and germinate precociously on the ear producing green seedlings. The *vp1* mutation affects only seed tissues. When rescued and transferred to soil prior to desiccation, mutant seedlings form a normal appearing mature plant. Interestingly, this mutation causes a pleiotropic phenotype. Besides displaying vivipary, *vp1* mutant seeds fail to accumulate anthocyanin pigments in embryo and aleurone tissues (Robertson, 1955). Consistent with this phenotype, activities of enzymes catalyzing anthocyanin biosynthesis were

not detectable in mutant seed tissues (Dooner, 1985). The lack of anthocyanin pigments is not likely to be a result of the reduced ABA-sensitivity of this mutant. ABA-deficient mutants accumulate normal amounts of anthocyanins, implying that ABA is not required for pigment formation. Furthermore, separation of the two phenotypes was observed in seeds carrying the *vp1-McWhirter* allele. Those seeds are unpigmented but non-viviparous (Coe et al, 1978). Therefore, the pleiotropic phenotype of the *vp1* mutant implies that in evolution, two processes, suppression of precocious germination and production of anthocyanins, have come under the control of a single protein.

As in ABA-deficient mutants, the viviparous phenotype of the *vp1* mutant is entirely determined by the genotype of the embryo (Robertson, 1955). Similarly, anthocyanin deficiency in embryo or aleurone solely reflects lack of functional VP1 in the respective tissue (Robertson, 1955). Thus, the failure to accumulate pigments in *vp1* mutant aleurone is not a direct or indirect result of precocious induction of germination. Cell autonomous function of VP1 in the aleurone was demonstrated in a transposable element-induced, somatically unstable mutant (*vp1-mum1*). Homozygous *vp1-mum1* seeds exhibit small sectors in the aleurone that have regained VP1 function due to excision of the *Robertson's Mutator* transposable element. These revertant sectors, recognizable by their pigmentation, can be as small as single cells, indicating that VP1 function does not result in production of a diffusable factor that might induce anthocyanins in neighboring cells (McCarty et al., 1989a).

The *Vp1* gene was cloned by transposon tagging using the *vp1-mum1* allele (McCarty et al. 1989a). It encodes a 2500 bp mRNA that is translated into a 73 kD protein. *Vp1* is expressed specifically in embryo and endosperm tissues of the developing seed. Within the endosperm, *Vp1* expression is likely to be restricted to the aleurone, as suggested from the low abundance of *Vp1* mRNA detected in whole endosperm extracts (McCarty et al. 1989a) and the apparent absence of a mutant phenotype in *vp1* mutant endosperm. The *Vp1* transcript is present in the seed as early as 10 days after pollination (DAP), reaches maximum accumulation at 16 DAP and decreases in abundance towards seed maturity. No *Vp1* expression was detected in germinating

seeds, root or shoot tissues (McCarty et al. 1989a; Carson, 1992). Hence, the expression pattern of *Vp1* is highly consistent with the seed-specific phenotype of the *vp1* mutant.

The *Vp1* gene consists of six exons and five introns. Apart from putative VP1 homologs cloned from barley, rice and *Arabidopsis* (M. Stoll and D.R. McCarty, unpublished results; Hattori et al., 1994; Giraudat et al., 1992), the sequence of VP1 shows no significant homologies to any known protein sequences, suggesting that VP1 is a novel protein. The N-terminus of VP1 is predicted to form two negatively charged amphipathic helices, a feature which is characteristic of many bacterial and eukaryotic transcriptional activators (Ptashne, 1988). Indeed, this region of VP1 was found capable of functionally replacing the acidic activation domain of the bacterial transcription factor GAL4 in a eukaryotic gene expression system (McCarty et al., 1991). This confirmed that the acidic region of VP1 has transcriptional activator function and suggested that VP1 may function as a regulatory protein in controlling seed maturation and anthocyanin accumulation.

In *Arabidopsis*, mutants displaying reduced sensitivity to ABA have been identified using genetic screens selecting for the ability of seeds to germinate on medium containing at least 3 μM ABA, a concentration that inhibits germination of wild-type seeds. In such screens, five loci controlling ABA-sensitivity have been identified: *Abi1*, *Abi2*, *Abi3* (Koorneef et al., 1984), *Abi4* and *Abi5* (Finkelstein, 1994). Mutations in any of these loci confer reduction in seed dormancy. However, while the phenotype of *abi3*, *abi4* and *abi5* mutants is restricted to seed tissues, *abi1* and *abi2* mutants are also impaired in stomatal regulation and a variety of stress responses in vegetative tissues (Koorneef et al., 1984; Finkelstein, 1994; Chandler and Robertson, 1994). Interestingly, when the *abi3-1* mutant was crossed to the ABA-deficient *aba* mutant, seeds of the resulting double mutant were desiccation intolerant, remained green and frequently displayed vivipary (Koorneef et al., 1989). The phenotype of the double mutant suggests that the *abi3-1* allele may be leaky and allow some ABA-responsiveness. Hence, additional reduction in seed ABA concentrations may be necessary to obtain a viviparous phenotype. Indeed, severe *abi3* mutants were isolated that were phenotypically similar to the *abi3-1/aba* double mutant,

confirming that a strong *abi3* allele conferring high insensitivity to ABA is sufficient to cause vivipary (Nambara et al., 1992; Ooms et al., 1993).

The *Abi3* gene was cloned by chromosome walking (Giraudat et al., 1992). The predicted protein of 79.5 kD displays discrete regions of sequence homology to the maize VP1 protein. Since there are also phenotypic similarities between the *abi3* and *vp1* mutant - at least with respect to seed-specific insensitivity to ABA - ABI3 and VP1 are likely to have similar functions in regulating ABA response in the seed. However, the functions of ABI3 and VP1 differ in so far that ABI3 is required for seed dormancy in *Arabidopsis* while VP1 does not impose seed dormancy in maize. Conversely, VP1, but not ABI3, induces synthesis of anthocyanins in the seed. Whether these phenotypic differences reflect differences in sequence between ABI3 and VP1 or the differential involvement of other factors remains to be determined.

The *Abi1* gene was cloned in two laboratories by chromosome walking (Leung et al., 1994; Meyer et al., 1994). At its C-terminus, the predicted ABI1 protein (47.5 kD) displays sequence similarity with the 2C class of serine-threonine protein phosphatases from rat and yeast. Its N-terminus exhibits features typical for a Ca^{++}-binding site (EF hand). Hence, ABI1 may function as a Ca^{++}-dependent protein phosphatase. Indeed, regulation of stomatal aperture by ABA involves Ca^{++} as a second messenger and protein phosphorylation events (Blatt and Thiel, 1993; Luan et al., 1993). How ABI1 may regulate ABA-induction of seed dormancy is thus far unknown.

Mutants affected in a thus far unknown mechanism

Three mutants of *Arabidopsis* (*lec1*, *lec2*, *fus3*) have been isolated that are non-dormant but normal in their response to ABA (Meinke, 1992; Keith et al., 1994; Meinke et al., 1994; Bäumlein et al., 1994). *lec1* and *fus3* have similar phenotypes. Immature mutant seeds germinate readily when placed in culture and display occasional vivipary when left to mature in the siliques. Furthermore, they are intolerant to desiccation and accumulate anthocyanins late in seed development, a feature that is not characteristic of wild-type seeds. Prematurely germinated seeds give rise to viable green seedlings that appear normal except that trichomes

are found on the adaxial surfaces of its cotyledons. Trichomes normally form only on leaves, stems and sepals, but not on cotyledons. Hence, *lec1* and *fus3* cotyledons are considered to be partially transformed into leaves, which gave two of the mutants their name (*lec*, "leafy cotyledons", Meinke, 1992). The *lec2* mutant also exhibits leafy cotyledons and accumulation of anthocyanin, but differs from *lec1* and *fus3* in that seeds are tolerant to desiccation and non-viviparous, and seedlings often appear distorted in shape (elongated hypocotyl, curled cotyledons) (Meinke et al., 1994). Unlike the *abi3* mutant, germination of *lec1* and *fus3* mutant seeds is inhibited by ABA, indicating that they retain normal sensitivity to ABA (Keith et al., 1994; Meinke et al., 1994). Proof for normal ABA synthesis in these mutants is still lacking. However, since ABA-deficient mutants have thus far not been shown to exhibit leafy cotyledons or accumulation of anthocyanins, it is unlikely that a possible lack of ABA would be the sole cause of the mutant phenotype. Nevertheless, the role of ABA in these mutants remains to be examined.

To investigate the interaction between *abi3* and leafy cotyledon mutants, double mutants were constructed. *abi3/lec1* and *abi3/fus3* double mutant seeds were highly viviparous, insensitive to ABA, exhibited leafy cotyledons and accumulated large amounts of anthocyanins (Meinke et al., 1994, Bäumlein et al., 1994). The additive effect of *abi3* and *lec1/fus3* in the double mutants suggests that *abi3* and *lec1* or *fus3*, respectively, are altered in distinct pathways. Consequently, suppression of precocious germination requires at least ABA, developmental factors controlling ABA-sensitivity and the leafy cotyledon-factors whose interactions with ABA, however, remain to be analyzed.

Analysis of Gene Expression

Late stages of seed formation are correlated with the expression of characteristic genes which were analyzed first and very extensively in cotton embryos (Galau et al., 1986, 1987; Hughes and Galau, 1991). Based on changes in the levels of specific sets of cotton mRNAs, late seed development has been categorized into several stages (Galau et al., 1991). The

earlier "maturation stage" comprises the longest time interval (19 days) and is characterized by high abundance of storage protein-mRNAs. This phase coincides with the presence of high concentrations of ABA. It is apparently terminated by abscission of the vascular connections between embryo and mother plant and is followed by the "postabscission stage" (5 days) during which maturation stage-specific mRNAs decline rapidly and a new set of mRNAs accumulates. To these postabscission stage-specific mRNAs belong the LEA's (this term was introduced by Galau et al., 1986) and the RAB's (*r*esponsive to *A*BA, a term used by other authors for similar proteins as LEA's). Subsequently, seed formation is terminated by rapid water loss and termination of transcription.

A similar temporal pattern of mRNA accumulation was reported for *Arabidopsis* (Parcy et al., 1994) and maize (Paiva and Kriz, 1994; Williams and Tsang, 1994). Nevertheless, for species other than cotton, late stages of seed formation are usually referred to as the "maturation phase" which is not subdivided into two stages. It should be mentioned that in maize, the seed maturation phase also correlates with the accumulation of anthocyanin pigments in embryo and aleurone tissues.

In the following, progress in our understanding of the regulation of maturation-specific genes (genes encoding storage proteins, LEA's and RAB's, proteins of the anthocyanin pathway) will be reviewed, placing emphasis on the roles of ABA and VP1 as regulators of seed maturation in monocot seeds.

Storage proteins

In many species, immature embryos cultured in ABA exhibited precocious and enhanced accumulation of storage proteins and their corresponding mRNAs as compared to those cultured on ABA-free medium or left to mature on the mother plant (Quatrano, 1987). These results indicate that ABA upregulates expression of storage protein genes. However, high levels of ABA are not required for expression of the major storage protein genes, as shown for maize and *Arabidopsis*. The *aba* mutant of *Arabidopsis* was found to accumulate normal levels of identified ABA-upregulated storage proteins (2S, 12S) (Koornneef et al., 1989) and their corresponding

mRNAs (Parcy et al., 1994). Similarly, maize ABA-deficient mutants accumulated mRNAs corresponding to the ABA-regulated 7S globulins in only slightly reduced amounts (Kriz et al., 1990; Paiva and Kriz, 1994). Hence, ABA does not normally appear to be a limiting factor in expression of these storage protein genes. In contrast, developing embryos of the ABA-insensitive mutants *vp1* of maize and *abi3* of *Arabidopsis* exhibited very low or undetectable expression of 7S globulins or 2S and 12S storage protein genes, respectively, indicating that VP1/ABI3 are required for their expression (Kriz et al., 1990; Paiva and Kriz, 1994; Nambara et al., 1992). Furthermore, exogenous ABA did not induce expression of storage protein genes in cultured immature *vp1* or *abi3* mutant seeds, while it did so in cultured immature wild-type seeds (Paiva and Kriz, 1994; Finkelstein and Somerville, 1990). Thus, VP1/ABI3 appear to be essential for ABA action. Since VP1/ABI3 are expressed at normal levels in ABA-deficient mutants (McCarty et al., 1991; Paiva and Kriz, 1994; Parcy et al., 1994), it may be that normal accumulation of storage proteins in these mutants is mediated by VP1/ABI3 either without a need for ABA or requiring residual amounts of ABA present in mutant seeds.

LEAs/RABs

An extensive survey in *Arabidopsis* examining the accumulation kinetics of 18 marker mRNAs expressed at high levels during mid to late seed development suggested that LEAs/RABs-encoding genes fall into distinct classes with different requirements for ABA and ABI3 to induce expression. For most markers, transcript levels did not solely correlate with the amounts of endogenous ABA or ABI3 present in the seed, thus implicating a role of other factors in controlling temporal patterns of expression (Parcy et al., 1994).

However, abundance of several tested mRNAs was highly reduced in seeds of the *aba* mutant as well as the *abi3* mutant (Parcy et al., 1994). Similarly in maize, expression of a well-studied LEA gene (*Em*) originally isolated from wheat was undetectable in developing seeds of mutants deficient for ABA or functional VP1 (McCarty et al., 1991). Hence, ABA as well as ABI3/VP1 appear to be required for expression of certain LEAs, which is consistent with a possible role of ABI3/VP1 in ABA perception or signal transduction in the seed.

To investigate further the interaction of VP1 and ABA in controlling expression of the *Em* gene in the maize seed, a transient gene expression system was used that is based on electroporation of maize protoplasts isolated from an immature embryo-derived suspension (Vasil et al., 1989). A plasmid containing the promoter (0.6 Kb) of the *Em* gene fused to the coding sequence of the bacterial β-glucuronidase (GUS) gene (Em-GUS; Marcotte et al., 1988) was used as a reporter construct. The VP1 cDNA was over-expressed from the constitutive CaMV 35S promoter enhanced by insertion of the first intron of the maize *Sh1* gene (Vasil et al., 1989) into the 5' untranslated leader of the VP1 cDNA (35S-Sh-VP1). In these experiments, electroporation of protoplasts with a mixture of Em-GUS and 35S-Sh-VP1 resulted in 100-300-fold higher GUS activity as compared to the very low Em-GUS activity detected in the absence of co-electroporated 35S-Sh-VP1 (McCarty et al., 1991). Similar activation was obtained when protoplasts electroporated with Em-GUS were cultured in ABA (McCarty et al., 1991), which is consistent with the reported ABA-regulation of Em-GUS in rice protoplasts (Marcotte et al., 1988; 1989). Over-expression of VP1 in maize protoplasts interacted synergistically with ABA, resulting in 2,500-fold induction of Em-GUS (McCarty et al., 1991). The synergistic effect of VP1 and ABA underlines the importance of both, VP1 and ABA, in high-level expression of *Em*. However, the substantial activation of Em-GUS obtained by either over-expressing VP1 or culture in ABA might imply that VP1 and ABA can partially activate Em-GUS independently. This would be in contrast to the absence of detectable *Em* transcript in *vp1* or *vp5* mutant embryos which contain normal levels of ABA or *Vp1* transcript, respectively (Neill et al., 1987; McCarty et al., 1991). However, action of endogenous ABA and VP1 that may be present in the wild-type protoplasts cannot be ruled out. To test this, Em-GUS was introduced into *vp1* and *vp5* mutant seed tissue (aleurone) via particle bombardment. In these experiments, ABA did not activate Em-GUS in *vp1/vp5* double mutant tissue while it did so in *VP1/vp5* tissue or when co-bombarded with recombinant VP1 (S. Cocciolone and D. R. McCarty, unpublished results), thus confirming that functional VP1 is required for ABA action. In *vp5* mutant tissue, over-expression of VP1 slightly activated Em-GUS, though 10-fold lower than in the presence of exogenous ABA.

This apparent independent activity of VP1 may be caused by low levels of maternal ABA present in *vp5* mutant seeds. Alternatively, the abnormally high levels of recombinant VP1 in expressing cells may allow some ABA-independent activation of the *Em* promoter normally not found in vivo.

The VP1 protein was subjected to functional analysis by testing deletion-derivatives for their ability to *trans*-activate Em-GUS. Sequence analysis and domain swapping experiments between VP1 and GAL4 had suggested that the N-terminus of VP1 contains an acidic transcriptional activation domain (McCarty et al., 1991). Indeed, deletion of this acidic domain abolished VP1's ability to activate Em-GUS. Replacing it with the acidic activation sequence of the herpes simplex virus transcription factor VP16 partially restored transcriptional activation of Em-GUS (McCarty et al., 1991). These results strongly indicate that VP1 functions as a transcriptional activator in inducing Em-GUS. Analysis of internal deletion-constructs of VP1 identified two highly basic domains that are important for activation of Em-GUS (L. Rosenkrans, V. Vasil, I.K. Vasil and D.R. McCarty, unpublished results).

Deletion analysis of the *Em* promoter indicated that two G-box-related sequences (Em1a: ACACGTGG; Em1b: ACACGTGC) which are conserved in many promoters responsive to ABA, light or anaerobiosis are involved in VP1- and ABA-mediated activation of *Em* (Marcotte et al., 1989; Guiltinan et al., 1990; V. Vasil et al., unpublished results). The finding that ABA-induction of *Em* does not require protein synthesis (Williamson and Quatrano, 1987) suggests that VP1 and ABA *trans*-activate *Em* directly through the G-box elements rather than through activation of intermediate regulatory genes further upstream in the ABA signal transduction pathway. Thus far, no DNA-binding activity of VP1 to these putative target sequences has been detected (T. Hattori, B. Li and D.R. McCarty, unpublished results). Hence, VP1 might activate *Em* via protein-protein interactions with G-box-binding protein(s). A bZIP-type protein binding specifically to the Em1a motif has been cloned (Guiltinan et al., 1990) and may thus be a candidate.

The molecular mechanism underlying the synergistic interaction of VP1 and ABA is thus far unclear. The possibility that ABA is required for high stability of the VP1 transcript or protein which then in turn activates *Em* is highly unlikely because in the ABA-deficient *vp5* mutant no reduction in VP1 transcript and protein levels was observed (McCarty et al., 1991; C. Carson and D.R. McCarty, unpublished results). Possibly, ABA post-translationally modifies the VP1 protein and thus enhances its *trans*-activation function. Alternatively, VP1 and an ABA-dependent factor might be part of a complex that forms on the *Em* promoter and induces transcription.

Genes encoding proteins of the anthocyanin pathway

As identified by mutants, at least eight genes are essential for accumulation of anthocyanin pigments in embryo and aleurone cells of the maturing maize seed (Fig. 1). Five of these genes (*A1, A2, C2, Bz1, Bz2*) encode enzymes of the anthocyanin biosynthesis pathway (Dooner et al., 1991). Expression of these structural genes requires the coordinate action of two regulatory proteins, C1 and a member of the R/B gene family (Coe et al., 1988). Both proteins exhibit features of transcription factors. The C1 protein contains a functional acidic transcriptional activation domain (Goff et al., 1991) and a region of sequence homology to the DNA-binding domain of animal *myb* proto-oncogene products (Paz et al., 1990). Proteins encoded by the R/B gene family display high homology with the helix-loop-helix motif of *myc* proto-oncogene products (Ludwig et al., 1989). At least for the promoter of the *Bz1* structural gene, it has been shown that sequences homologous to the consensus binding sites of animal MYB and MYC proteins are essential for C1- and R-mediated activation of *Bz1* expression (Roth et al., 1991), suggesting a direct interaction of C1 and R with these sequences in the *Bz1* promoter.

A third regulatory factor required for pigmentation of tissues in the developing maize seed is VP1. The lack of anthocyanin pigments in *vp1* mutant seed is associated with the absence of *C1* transcript (McCarty et al., 1989a). Over-expression of a 35S-C1 construct in *vp1*

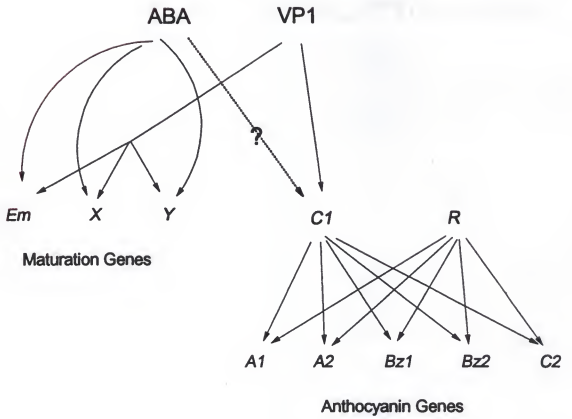


Fig. 1. Role of VP1 in activation of seed maturation-related pathways and anthocyanin biosynthesis.

mutant aleurone cells by particle bombardment complemented the failure to accumulate anthocyanins in a cell autonomous fashion (Hattori et al., 1992). Thus, lack of C1 appears to be responsible for the block in anthocyanin synthesis in *vp1* mutant seed. A direct role of VP1 in activating *C1* expression was concluded from the demonstration that over-expression of VP1 in maize protoplasts activated transcription of a *C1* promoter-GUS fusion gene. Most importantly, VP1 function in activating *C1* was dependent on its transcriptional activation domain (Hattori et al., 1992). Hence, VP1 and C1 are part of a regulatory hierarchy controlling activation of anthocyanin structural genes.

Several lines of evidence indicate that VP1's function in activating *C1* is distinct from its function in activating *Em*. First, though induction of both genes is dependent on the acidic activation domain of VP1 and thus appears to involve transcriptional activation, other domains of VP1 involved in function differed depending on the target promoter. While sequences in the middle of the VP1 protein were required for *trans*-activation of Em-GUS in maize protoplasts, VP1-activation of C1-GUS was dependent on the C-terminal end of VP1 (L. Rosenkrans, V. Vasil, I.K. Vasil and D.R. McCarty, unpublished results). This is consistent with the non-viviparous/unpigmented phenotype of the *vp1-McWhirter* mutant which produces a truncated VP1 protein lacking ca. 150 bp from the C-terminus (McCarty et al., 1989b).

Second, in agreement with the involvement of distinct domains of VP1, different *cis*-elements in the *C1* and *Em* promoters appear to be the target of VP1 function. In contrast to activation of *Em* which depended on two G-box sequences, activation of *C1* did not require any of the two G-box-like sequences present in the promoter but the 13 bp sequence -145 TCCATGCATGCAC -158 (Hattori et al., 1992). This sequence, designated as Sph-element, is found in promoters of other seed-specific genes (Dickinson et al., 1988).

Finally, VP1-mediated activation of *Em* and *C1* differ in their interaction with ABA. While there is a synergistic effect of VP1 and ABA in activating *Em*, the role of ABA in VP1-mediated activation of *C1* is less clear. Anthocyanins accumulate at normal levels in ABA-deficient mutants of maize, suggesting that *C1* expression is hormone-independent. On the

other hand, ABA *trans*-activated C1-GUS in maize protoplasts. Moreover, a *c1* mutant (*c1-p*; Chen and Coe, 1978) that fails to accumulate anthocyanin during seed development carries a 5 bp deletion in the promoter region of the gene that, when reconstructed by site-directed mutagenesis of the *C1* promoter and used in transient expression experiments, specifically abolished ABA-responsiveness without severely affecting *trans*-activation by VP1 (Hattori et al., 1992). Hence, the unpigmented phenotype of *c1-p* may be caused by a deficiency in ABA-response. Pigmentation in ABA-deficient mutants might be possible if activation of *C1* has an ABA-requirement several orders of magnitude lower than activation of *Em*.

The Aleurone Germination Response in Cereal Seeds

During cereal seed development, the outermost endosperm cells differentiate into the aleurone layer which at seed maturity consists of small, thick-walled cells with plasmodesmatal connections. The aleurone cells are characteristically rich in protein and lipid bodies, mitochondria and ER, but are devoid of starch grains. In response to GA released by the germinating embryo, these highly specialized cells synthesize large amounts of hydrolytic enzymes co-translationally on the rough ER and - following proper folding in the lumen of the ER - secrete these enzymes into the endosperm. This aleurone germination response to GA can be inhibited by treatment with ABA.

The predominant hydrolytic enzyme synthesized is α-amylase, constituting ca. 15-20% of total translatable mRNA and ca. 30% of total protein synthesis in germinating barley seeds (Khursheed and Rogers, 1988). In barley and wheat, the major source of α-amylase is the aleurone layer, whereas in maize, sorghum and rice, significant contributions are also made by the embryo scutellum (Ranki and Sopanen, 1984; Dure, 1960a,b; Akazawa and Miyata, 1982).

Hormonal Regulation

Extensive studies in barley and several investigations with other cereal species have shown that synthesis of cereal α-amylases is induced by GA and antagonistically inhibited by ABA (for review see: Jacobsen and Chandler, 1987; Jones and Jacobsen, 1991; Fincher, 1989). The first reports on GA-induced α-amylase activity in germinating barley seeds appeared in 1960 (Paleg, 1960a; b; Yomo, 1960). The action of ABA as an antagonist of GA was discovered in 1966 (Chrispeels and Varner, 1966).

Application of transcription and translation inhibitors indicated that the GA-induced appearance of α-amylase activity was due to de novo synthesis of the enzyme (Varner and Chandra, 1964; Filner and Varner, 1967). Ultimate proof for an effect of GA and ABA on α-amylase synthesis came from the demonstration that GA treatment drastically increased the amount of in vitro translatable α-amylase mRNA (Higgins et al., 1976), while simultaneous application of ABA blocked this effect (Mozer, 1980). Run-on transcription experiments provided evidence that GA and ABA regulate the transcription of α-amylase genes (Jacobsen and Beach, 1985; Zwar and Hooley, 1986). Eventually, the development of transient gene expression technology has provided an additional tool to elucidate GA and ABA action. It was shown that transient expression of a wheat α-amylase promoter-GUS reporter gene fusion construct in oat aleurone protoplasts was regulated in the same manner as the endogenous genes (Huttley and Baulcombe, 1989).

Relatively few studies have addressed hormonal regulation of maize α-amylase. Ingle and Hageman (1965) reported a stimulating effect of GA on catabolism of carbohydrates in excised endosperms. In a different study (Harvey and Oaks, 1974), exogenous GA applied to excised endosperms further increased (3-fold) total amylase activity in germinating seeds of a GA-deficient mutant (*d5*), but not in wild-type seeds. In contrast, culture of wild-type endosperms in ABA strongly reduced amylase activity. Even though no molecular data are available, these results indicate that maize α-amylase genes are probably regulated by GA and

ABA in a similar fashion as other cereal α-amylases. Ultimate proof for this, however, is still lacking.

It has now been generally accepted that the embryo is the site of GA biosynthesis in the germinating barley grain. When de-embryonated seeds are imbibed, no increase in GA levels in the endosperm and very little subsequent production of α-amylase can be detected (Jacobsen and Chandler, 1987). Further studies demonstrated that the scutellum, rather than the embryo axis, is the source of GA (Radley, 1967; MacLeod and Palmer, 1967).

In maize, evidence for the importance of the germinating embryo as a source of GA has been contradictory. In imbibed de-embryonated seeds, Dure (1960) found only β-amylase (which is stored in protein bodies in the dry seed and therefore is not de-novo synthesized during germination), but no α-amylase activity, whereas whole kernels showed both activities. Two other studies (Harvey and Oaks, 1974; Goldstein and Jennings, 1978), however, demonstrated comparable total amylase activities in de-germed as well as whole seed endosperm. As only part of the activity was due to release of β-amylase from protein bodies, it was concluded that mature seeds store considerable amounts of GAs in the endosperm. Therefore, the germinating maize embryo does not appear to be an essential source of GA for α-amylase synthesis in the aleurone.

The α-Amylase Genes

α-amylases of cereals can be biochemically separated into a number of isoforms that differ in their isoelectric point (pI) but not considerably in their molecular weight. In barley, there are two families of isozymes, the low-pI α-amylases with pIs of ca. 4.4-5.2 and the high-pI α-amylases with pIs of ca. 5.7-6.2 (Jacobsen and Chandler, 1987). These two families differ in many other biochemical characteristics while isozymes within those families are more alike. Though some of the variants are post-translational modifications of the same gene product, a genetic basis for most of the variation seen became evident when the gene(s) for the low and high pI families were mapped to different chromosomes, chromosome 1 and 6, respectively

(Brown and Jacobsen, 1982; Muthukrishnan et al., 1984). Isolation and sequencing of a number of cDNA clones verified that there are sequence differences between isoforms. Base sequence homology is 90-95% within gene families and about 75% between gene families (Jacobsen and Chandler, 1987). Southern blot analyses of two barley varieties revealed that there are at least 6-7 high-pI genes and at least 3 low-pI genes, indicating that α-amylases are encoded by two multigene families (Khursheed and Rogers, 1988; Muthukrishnan et al., 1984). While no detailed mapping has been undertaken in barley, three rice α-amylase genes were found to be clustered within 28 kb of genomic DNA. Molecular analysis suggested gene duplication as a cause (Sutliff et al., 1991).

The two barley α-amylase gene families are regulated differently in the aleurone of germinating seeds. mRNA levels of the high-pI isoforms increase very quickly upon imbibition, reaching a maximum after two days and decreasing to low levels after four days. Synthesis of low-pI isoform-mRNAs begins later, not before three days after imbibition, but then increases rapidly so that low-pI isozymes become the dominant enzyme group after four days of imbibition (Chandler and Jacobsen, 1991). The two isoforms are also differentially responsive to GA. In some studies with isolated aleurone layers, mRNA as well as protein of the low-pI isoforms can be detected before GA is added, while those of high pI-isoforms cannot (Chandler and Jacobsen, 1991; Jacobsen and Higgins, 1982; Rogers, 1985). Others, using isolated aleurone layers or aleurone protoplasts, find no low-pI message in the absence of GA (Chandler and Jacobsen, 1991; Nolan and Ho, 1988). However, low-pI isoforms appear to be more sensitive to GA as they respond to GA-concentrations as low as 10^{-9} M (Nolan and Ho, 1988). Thus, low-pI α-amylase genes might be either more responsive to GA or leaky in expression.

In comparison to barley α-amylases, many fewer studies on maize α-amylases have been reported. Partial purification of α-amylases from endosperm of germinating seeds has revealed two major groups of isozymes, one with pIs of 5.1-5.7, the other with pIs of about 4.6 (Warner and Knutson, 1991). Other authors report the purification of α-amylase isozymes with a variety of pIs (Warner et al., 1991; MacGregor et al., 1988; Chao and Scandelios, 1971). No

genes coding for α-amylases have been cloned so far. Thus no information about gene expression is available.

The Organization of α-Amylase Promoters

Gene expression is thought to be regulated by proteins ("*trans*-acting factors") that bind in a sequence-specific manner to short stretches of base pairs ("*cis*-acting elements") located in the promoter region of the gene. With an interest to study regulation of α-amylase gene expression, genomic clones were isolated. Sequence comparisons revealed little homology between promoters of α-amylase genes belonging to different pI groups which may relate to their differential expression in response to hormones described above. However, a few blocks of sequence were found highly conserved among barley, wheat and rice α-amylase promoters:

High-pI, barley CGCCTTTTGAGCTCACCGTACCGGCCGATAACAAACTCCGGCCGACATATCCACTG -117
(Khursheed and Rogers, 1988)

Low-pI, barley GCACCTTTTCTCGTAACAGAGTCTGGTATCCATGCA -98
(Whittier et al., 1987)

Low-pI, wheat GCACCTTTTTTCGTAACAGAGTCTGGTATCCATGCA -95
(Huttley et al., 1992)

To identify *cis*-acting elements involved in hormone-regulated expression, functional analyses of α-amylase promoters have been performed. For this purpose, mutated promoter sequences are fused to a reporter gene (e.g. GUS, Luciferase) and are assayed for function in a transient gene expression system (electroporation of aleurone protoplasts or particle bombardment of intact aleurone layers).

Progressive 5' truncations of α-amylase promoters showed that 289 bp of a wheat low-pI α-amylase promoter (Huttley and Baulcombe, 1989) and 174 bp of a barley high-pI α-amylase promoter (Jacobsen and Close, 1991) were sufficient to direct GA- and ABA-regulated

expression of a reporter gene, indicating that *cis*-acting elements are positioned in the proximal region of the promoters. Indeed, Skriver et al. (1991) demonstrated that a chimeric construct containing 69 bp (-189 to -120) of the barley high-pI promoter fused to the 35S TATA box could impose increased transcription by GA and its suppression by ABA. Moreover, six tandemly repeated copies of the sequence GGCCGATAACAAACTCCGGCC (21 bp) conferred proper GA- and ABA-regulation. However, this result could not be confirmed when particle bombardment was used as the method of transformation (J. Rogers, pers. communication; U. Hoecker, unpublished results) suggesting that other *cis*-elements apart from TAACAAA contribute to GA-regulated transcription. This was confirmed when clustered point mutations were introduced covering the proximal region of the promoters. Mutations in the pyrimidine box (CCTTTT) or in the TATCCAC/T box reduced GA-induced transcription to about 20 % of minimal level in the barley high-pI and low-pI promoters (Gubler and Jacobsen, 1992; F. Gubler, pers. communication; Lanahan et al., 1992). Thus the entirety of the three conserved elements appears to be involved in mediating GA-response. Interestingly, in no case could ABA-responsive elements be separated from GA-responsive elements, suggesting that GA and ABA function through the same *cis*-elements in the α-amylase promoters.

Rogers and co-workers (Lanahan et al., 1992) identified an additional element in the low-pI promoter that is located between positions -152 and -134, just upstream of the pyrimidine box. Mutations in this element reduced the level of expression by 96% while retaining significant but low GA-responsiveness. This region of the promoter (termed "O2S" element) shows sequence homology to two well-described motifs: the "endosperm box", a conserved element present in promoters of maize, barley and wheat endosperm protein genes, and the consensus sequence for binding of the maize Opaque-2 protein which is a leucine zipper protein (bZIP) that is necessary for transcription of the 22 kDa zein genes. Interestingly, it was found that substitution of the GA-responsive TAACAGA sequence of the low-pI promoter with an ABA response element (ABRE) from the rice *Rab-16A* gene converted the promoter from a GA-upregulated one into one whose transcription was increased by ABA (Rogers and Rogers, 1992). Thus, the ABRE

regulated transcription in the context of the low-pI α-amylase promoter in a similar way as it does in its native rice promoter. Importantly, its function in the amylase promoter was highly dependent upon the presence of the O2S sequence. Thus, the O2S element appears to function as a "coupling element" that is necessary for high-level, hormone-regulated transcription from the low-pI α-amylase promoter (Rogers and Rogers, 1992). No O2S-like sequence is evident in high-pI α-amylase promoters. However, inserting the O2S element from a low-pI promoter into a high-pI promoter at a position upstream of the pyrimidine box enhanced transcription ca. 5-fold, suggesting that the O2S element function could interact properly with the high-pI promoter fragment to give high-level transcription (Rogers et al., 1994).

To identify the *trans*-acting factors that bind to the *cis*-elements in the promoters and thereby confer hormone-dependent expression of the α-amylase genes, DNA-protein interactions have been characterized using band shift assays and DNase I footprinting analyses. In a wheat low-pI promoter (α-Amy 2/54), the O2S box and the TAACAGA element, but not the TATCCAC sequence, were found protected from DNase I digestion, confirming the binding of protein factor(s) to at least two functionally important elements. However, these binding activities were not dependent upon GA (Rushton et al., 1992). Evidence for the presence of a GA-dependent factor on a barley low-pI promoter was provided by Sutliff et al. (1993). Results from band shifts performed in this study demonstrated that a GA-inducible binding activity interacted with the TAACAGA and TATCCAC elements in a sequence-specific manner. In a different report on a rice α-amylase promoter (*Amy3c*), GA-dependent binding to a pyrimidine box-like sequence was demonstrated (Goldman et al., 1994). However, since this promoter displays little sequence homology to barley and wheat promoters, comparisons cannot be made. In summary, GA-independent as well as GA-dependent factors appear to constitute the complex(es) on α-amylase promoters that result in GA-regulated expression. Further characterization of the protein-DNA and protein-protein interactions is needed.

GA and ABA Signal Transduction

It is generally accepted that molecules with hormonal function act as ligands that upon binding to their specific receptors elicit a response which ultimately can result in altered gene expression. In animals, a large number of hormone receptor-encoding genes has been cloned and characterization of their gene products has demonstrated that receptors can be found located intracellularly (e.g. steroid hormone receptors) or integrated into the cell membrane with their ligand-binding domain facing the extracellular space. Identification of plant hormone receptors has proven difficult. Though hormone-binding proteins have been identified, definite proof for the function of these proteins is still lacking.

With respect to GA and ABA signal perception in the cereal aleurone, studies have so far concentrated on the identification of the cellular site of the receptors. Results from at least one study suggest that GA does not have to enter the cell to regulate gene expression. Hooley and colleagues (Hooley et al., 1991) demonstrated that GA immobilized to Sepharose beads was capable of enhancing α-amylase transcription in oat aleurone protoplasts. Though these data point to a perception of the GA signal on the external surface of the plasma membrane, the existence of intra-cellular receptors cannot be ruled out. Clear evidence against intra-cellular receptors came from elegant experiments performed in the laboratory of R. L. Jones. A method was developed to visualize α-amylase gene expression and α-amylase secretion from individual protoplasts (Hillmer et al., 1992). This allowed to test whether or not hormones microinjected into the cytosol of aleurone protoplasts were capable of eliciting a response (Gilroy and Jones, 1993). It was found that protoplasts injected with GA did not respond to the hormone. Similarly, ABA microinjected into protoplasts was ineffective in antagonizing the stimulating effect of pre-applied external GA. The failure to respond to microinjected hormones was not due to disruption of protoplast function by microinjection since protoplasts that had been subjected to this procedure remained responsive to externally applied GA. These results indicate that the sites of perception of GA and ABA are located on the external face of the plasma membrane in aleurone cells.

There is little concept of how the perceived GA and ABA signals are transduced from the receptors to the nucleus. The function of Ca^{++} and calmodulin (CaM) as second messengers that regulate protein kinase activity has been characterized in many animal and a few plant systems (Roberts and Harmon, 1992; Neuhaus et al., 1993). Also in barley aleurone protoplasts, treatment with GA was found to increase cytoplasmic Ca^{++} and CaM concentrations (Gilroy and Jones, 1992, 1993). ABA reversed the effect of GA on $[Ca^{++}]_i$ (Gilroy and Jones, 1992). Even though the increase in $[Ca^{++}]_i$ and [CaM] preceded the GA-induced increase in α-amylase activity by 2-4 h (Gilroy and Jones, 1992), direct evidence for an involvement of Ca^{++} and CaM in the regulation of α-amylase transcription is still lacking. Ca^{++} and CaM have been found to regulate the activity of a slow vacuolar ion channel located in the tonoplast of storage protein vacuoles in barley aleurone cells (Bethke and Jones, 1994). Moreover, CaM was shown to stimulate Ca^{++} uptake into the ER where the Ca^{++} containing α-amylase enzyme is synthesized (Bush et al., 1993). Thus, hormone-regulated changes in Ca^{++} and CaM concentrations may be regulating processes such as α-amylase formation and secretion rather than having a direct effect on the transcription of α-amylase genes.

Analysis of mutants provides a valuable tool to study the genetics underlying the regulation of hormone action. Many mutants have been isolated that display altered responses to GA, suggesting that these mutants are affected in a component of GA signal transduction. Since GA promotes stem and leaf elongation, these mutants have been identified by their altered plant height. They fall into two classes: 1) those that show a reduced sensitivity to GA ("GA-insensitive mutants") and are therefore of dwarf stature, and 2) those that show an enhanced sensitivity to GA ("constitutive response mutants") and are therefore excessively tall. In response mutants, the concentrations of biologically active GAs do not in accord with the phenotype. Generally, GA-insensitive mutants accumulate higher concentrations of active GAs as compared to wild-type, while tissues of constitutive response mutants contain reduced GA concentrations (Stoddart, 1984; Fujioka et al, 1988; Croker et al., 1990). These observations

have been attributed to feedback regulations on GA metabolism in response to altered GA-sensitivity.

To the class of GA-insensitive mutants belong the *Rht* (reduced height) mutants of wheat. A total of 10 *Rht* loci have been identified, showing varying degrees of dominance. Of these, the mutation *Rht3* exerts the strongest dwarfing effect. The GA-insensitive phenotype of *Rht3* is also expressed in the aleurone: germinating seeds had 75% reduced levels of amylase activity as compared to tall (*rht*) varieties and showed no or little increase in amylase activity after GA treatment (Gale and Marshall, 1975; Fick and Qualset, 1975). The degree of GA-insensitivity of the aleurone was found to increase with the dosage of *Rht3* alleles (Gale and Marshall, 1975). A similar GA-insensitive mutant has been described in rice (Mitsunaga et al., 1994). The finding that the failure to respond to GA is expressed in plant and seed tissues indicates that these tissues share at least in part a common signal transduction pathway.

Two dominant GA-insensitive dwarf mutants have been identified in maize (*D8, D9*). Besides being of reduced stature, these mutants mimic additional characteristics of GA-deficient mutants, such as reduced apical dominance and formation of anthers on the ear (Coe and Neuffer, 1977). *D8* and *D9* are located on 1L and 5S, respectively (Coe and Neuffer, 1977). Since the region of 5S contains duplicate loci with 1L it is likely that *D8* and *D9* are duplicate loci encoding gene products with identical or similar function. X-ray-induced chromosome breakage was used to create clonal sectors of wild-type cells within *D8* mutant tissue. Results from these experiments indicated that *D8*-mediated effects can be expressed cell autonomously at least in some tissues (Harberd and Freeling, 1989) which is consistent with the hypothesis that the wild-type gene product is part of a GA signal transduction pathway. The gain-of-function nature of the mutation in association with a GA-insensitive phenotype allows to speculate about the function of the wild-type gene product. Possibly, *d8*(+) (and *d9*(+)) encodes a negative regulator of GA response that normally is inactivated by exposure of the cell to GA. In this scenario, the mutant *D9* protein would be constitutively active in this repressor activity, i.e. even in the absence of GA (Harberd and Freeling, 1989).

A constitutive response mutant has been identified in barley (Foster, 1977). This recessive mutation, termed "*slender*" (*sln*), causes a plant to appear as if it had been treated with high doses of GA (Lanahan and Ho, 1988; Croker et al., 1990). Also, α-amylase genes were highly expressed in *sln* mutant half grains in the absence of applied GA (Chandler, 1988). Thus, the absence of functional SLN protein causes constitutive expression of GA-responses and thereby uncouples transcription of GA-regulated genes from a need for GA. This phenotype suggests that *Sln* encodes a negative regulator of GA-response. Importantly, α-amylase production in *sln* mutant aleurones was susceptible to inhibition by ABA, indicating that the *sln* mutant retains normal sensitivity to ABA (Chandler, 1988; Lanahan and Ho, 1988). Hence, ABA most probably functions at a step downstream of GA in the signal transduction pathway leading to regulation of α-amylase transcription. A function of ABA fully independent of GA cannot be ruled out but is unlikely because GA and ABA appear to act through the same response elements in α-amylase promoters. The findings also indicate that GA and ABA do not act at the same site in the signal transduction pathway, i.e. they do not for example antagonistically phosphorylate/ de-phosphorylate an intermediate.

The Developmental Switch from Seed Maturation to Seed Germination

Desiccation is the normal terminal event in seed development, leading to a state of metabolic quiescence. In many species (e.g. maize, bean), hydration of the mature, dry seed is sufficient to initiate germination. Thus, in these seeds (termed quiescent or non-dormant seeds), the transition from seed maturation to germination is associated with the reversal of the desiccated state. Seeds of other species (e.g. cereals, *Arabidopsis*) develop dormancy during late stages of seed development. In these species, freshly harvested mature seeds do not germinate following imbibition but require a treatment such as light, low temperature or after-ripening (dry storage) to overcome the state of dormancy and allow induction of germination.

Hence, dormant seeds execute the developmental switch to germination during the imposed dormancy-breaking treatment.

As described earlier, the transition from seed development to germination is associated with major changes in gene expression. It is generally accepted that in quiescent seeds, maturation-related genes cease expression once the water content falls below a level permitting transcriptional activity, and following imbibition, expression of a new set of genes is initiated which is specific to the germinating seed. What gene expression programs are executed in imbibed, dormant seeds is less clear. However, there is evidence from studies in wheat pointing to a maintenance of maturation-specific gene expression during the state of dormancy (Ried and Walker-Simmons, 1990, 1993; Morris et al., 1991).

Most importantly, maturation and germination programs appear to be regulated coordinately in the developing seed. Not only is precocious germination of the immature embryo suppressed, but similarly, the premature induction of germination-related genes appears to be inhibited during this developmental state. Developing seeds of wheat and barley contain biologically active GAs in concentrations adequate to induce α-amylase production in the aleurone layer (Wheeler, 1972; Radley, 1976). Nevertheless, only very low levels of α-amylase enzyme activity or mRNAs were detected in immature seeds (Comford et al., 1986; Garcia-Maya et al., 1990). More compelling, neither α-amylase activity nor α-amylase gene expression was induced when immature seeds were exposed to exogenous GA (Nicholls, 1979; Comford et al., 1986; Garcia-Maya et al., 1990; Oishi and Bewley, 1990). Similar results were obtained when treating dormant seeds with GA (Schuurink et al., 1992a). Given that immature or dormant embryos excised from the seed and placed in culture are capable of responding to GA, these data are strong evidence for active repression of the GA-response in developing or dormant seeds. Results consistent with this idea were also reported for dicot seeds (soybean, castor bean). In these species, immature seeds contained enzymes involved in the degradation of fatty acids and proteins (malate synthetase, LeuNase, isocitrate lyase) at a much lower level than germinating seeds or isolated embryos in culture (Kermode, 1990).

The mechanism underlying the developmental switch from seed maturation to seed germination, precisely the "turning off" of maturation-related genes and the de-repression/ induction of germination-specific genes, is only poorly understood. Evidence on the molecular nature of this switch is reviewed in the following for quiescent and dormant seeds.

Quiescent Seeds

Desiccation and subsequent rehydration of the seed appears to be the normal trigger to switch the developmental program from maturation to germination (Comai and Harada, 1990). Even when applied prematurely, drying resulted in the termination of maturation-related gene expression (Oliver et al., 1993) and, upon imbibition of the dry seed, the induction of genes specifically associated with germination (Kermode, 1990). Drying altered the developmental potential of seeds such that α-amylase production became sensitive to GA (Evans et al., 1975; Nicholls, 1979; Armstrong et al., 1982; Comford et al., 1986; Oishi and Bewley, 1990).

The nature of this switch in GA-sensitivity remains elusive. King (1976) has postulated that accumulation of ABA in the developing seed prevents precocious induction of hydrolase gene expression in the developing aleurone. Indeed, drying has been shown to cause a concomitant decline in grain ABA content (McWha, 1975; King, 1976; Oishi and Bewley, 1990). Moreover, incubation of immature grains in buffer which caused a drop in endogenous ABA to undetectable levels evoked GA-responsiveness of the aleurone (Napier et al., 1989). Hence, depletion of endogenous ABA, by drying or washing, may be responsible for the induction of GA-responsiveness. This is consistent with the finding that in maize mutants that are either deficient for embryonic ABA (*vp5*) or insensitive to ABA (*vp1*), α-amylase activity was induced late in seed development (Wilson et al., 1973).

However, results from Oishi and Bewley (1990) indicate that induction of α-amylase synthesis as a result of drying is not solely due to a reduction in ABA content. The authors compared the responses of maize kernels to premature drying and treatment with an ABA biosynthesis inhibitor (flouridone) which reduces ABA contents in the seed to a similar extent as

drying of immature seeds and elicits precocious germination of immature maize kernels. If drying merely served to deplete endogenous ABA in developing seeds, then flouridone-treated kernels and dried seeds should behave similarly with respect to GA-response. However, while drying resulted in synthesis of high levels of α-amylase following imbibition, flouridone-treated seeds produced only very low amounts of α-amylase in response to GA. Hence, drying may achieve two effects: 1) it frees seed tissues of the inhibitory effect of ABA, and 2) it renders the aleurone competent of responding to GA. The cause of the ABA-independent GA-insensitivity in immature seeds is thus far unknown.

Dormant Seeds

Cereals

Imposed dormancy in cereal species is normally released by prolonged storage of dry seeds (afterripening). The duration of seed dormancy following seed maturity depends on a variety of factors such as the genetic constitution (cultivar), the environmental conditions during grain maturation (low temperatures and short day length increase dormancy; Schuurink et al., 1992b) and the rehydration temperature (high temperatures enhance dormancy; George, 1967). Such differences in the depth of seed dormancy have been utilized to investigate the roles of ABA concentration and ABA-sensitivity in preventing embryo germination. No clear correlation between ABA content in the mature embryo and the degree of dormancy was found (Walker-Simmons, 1987, 1988; Morris et al., 1989; Skadsen, 1993). However, there are large differences between dormant and non-dormant embryos with respect to sensitivity to ABA, as measured by the capacity of ABA to inhibit germination. Isolated embryos of a non-dormant wheat cultivar lost their sensitivity to ABA in culture as the grain entered maturation stage, whereas those of a dormant cultivar retained sensitivity to ABA beyond grain maturity (Walker-Simmons, 1987). Similarly, elevating the incubation temperature from 15°C to 30°C, thus inducing high-temperature dormancy, significantly enhanced the ability of ABA to block germination of isolated

wheat embryos (Walker-Simmons, 1988). This differential inhibitory effect of ABA depending on the degree of dormancy was also observed using intact, mature seeds (Morris et al., 1989).

In conclusion, depth of dormancy appears to be positively correlated with ABA-sensitivity with respect to inhibition of germination. Why, and if, enhanced ABA-sensitivity is the immediate cause for inhibition of germination is unclear. Because ABA has been shown to inhibit water uptake by the embryo (Schopfer and Plachy, 1984), it has been suggested that high sensitivity to ABA in dormant seeds may result in reduced water uptake in the embryo and thereby prevent radicle emergence (Walker-Simmons, 1987). Additionally, ABA may have a differential effect on gene expression in dormant and non-dormant seeds. Indeed, transcript levels of a variety of ABA-responsive genes remained high in imbibed dormant wheat seeds, whereas they declined rapidly in non-dormant seeds following imbibition (Morris et al., 1991). Similarly, maturation-related LEA proteins were abundant in rehydrated dormant seeds but not in non-dormant seeds (Ried and Walker-Simmons, 1990, 1993). However, most of the identified ABA-responsive proteins that accumulate specifically in dormant seeds are predicted to be dehydrins and may therefore function primarily in maintaining the embryo in a desiccation-tolerant state rather than in directly inhibiting germination.

Exogenous application of ABA is known to inhibit GA-mediated activation of hydrolase genes in the aleurone of germinating cereal seeds (Jacobsen and Chandler, 1987). In this context, the following observation may be important. Imbibed dormant barley seeds showed very low expression of α-amylase genes as compared to non-dormant seeds (Morris et al., 1991; Schuurink et al., 1992a; Skadsen, 1993). Moreover, dormant grains produced less α-amylase in response to GA than non-dormant grains (Schuurink et al., 1992a; Skadsen, 1993). Hence, seed dormancy appears to be correlated with a reduced responsiveness of the aleurone to GA. Experiments with isolated aleurone layers indicated that the reduced GA-sensitivity of aleurone cells of dormant barley seeds is dependent on the presence of the starchy endosperm (Schuurink, 1992a; Skadsen, 1993), implying that the starchy endosperm may liberate an inhibitory factor that diffuses to the aleurone cells. It is suggestive that this putative diffusible

factor may be ABA stored in the dry seed. However, since the dormant and non-dormant seeds used in one experiment contained similar concentrations of ABA (Skadsen, 1993), it may be that the higher ABA-sensitivity in dormant seeds relative to non-dormant seeds plays a role in inhibiting GA-response in the aleurone. However, it cannot be excluded that a factor(s) other than ABA inhibits the GA-response in the aleurone of dormant seeds. α-amylase genes are known to be sensitive to repression by soluble carbohydrates (Yu et al., 1991; Karrer and Rodriguez, 1992). Possibly, aleurone cells of dormant seeds display a higher sensitivity to the inhibitory effect of soluble sugars supplied by the starchy endosperm. Alternatively, the inhibitory factor may be synthesized in the aleurone cells themselves and the presence of the starchy endosperm is only required to provide an environment of high osmolality which may be essential to maintain production of the putative inhibitor of GA-response in the aleurone cells.

Arabidopsis

Dormant seeds of *Arabidopsis* require either several months of dry storage or rehydration followed by exposure to low temperatures and light in order to break dormancy and induce germination. Analyses of mutants has demonstrated that initiation of dormancy during late seed development involves the action of ABA. Even in light, wild-type seeds are normally incapable of germinating during the seed maturation phase and, for a period of time, after reaching seed maturity. In contrast, seeds of the ABA-deficient mutant *aba* gradually acquire germination capacity during seed development and at maturity germinate at a frequency of 100% in light and 30% in darkness (Karssen et al., 1983; Karssen and Lacka, 1985). Hence *aba* mutant seeds display highly reduced dormancy. A germination behavior similar to *aba* mutant seeds was observed for the ABA-insensitive mutants *abi1*, *abi2* and *abi3* (Koorneef, 1984), suggesting that these mutants are non-dormant due to a failure to respond to ABA. However, as mentioned earlier, strong alleles of *abi3* do not only cause lack of seed dormancy but also vivipary, whereas seeds carrying strong alleles of *aba* have thus far not been shown to be viviparous. Thus, an ABA-independent function of ABI3 cannot be ruled out.

While ABA is clearly involved in the induction of seed dormancy, its role in the maintenance of a dormant state beyond seed maturity is less clear. Late in seed development, ABA concentrations decline rapidly to a very low amount present in the dry seed. This amount has been considered insufficient to inhibit germination (Karssen et al., 1983). Also, *aba* mutant and wild-type seeds were equally sensitive to applied ABA (Koornneef et al., 1982, 1984), indicating that the difference in germination capacity between *aba* mutant and wild-type seeds is not due to a difference in ABA-sensitivity. It was therefore thought unlikely that ABA or ABA-sensitivity are involved in maintaining the state of dormancy (Karssen and Lacka, 1985). Instead, Karssen and Lacka (1985) proposed that the maintenance of dormancy is, at least in part, mediated by an insensitivity of the seed to GA. This was concluded from evidence showing that a gradual relief of dormancy by afterripening, cold or light treatments was correlated with an increased sensitivity of the seed to the germination-promoting effect of applied GA (Karssen and Lacka, 1985; Derkx and Karssen, 1993). GA is normally absolutely required for induction of seed germination, as evident from the fact that seeds of the GA-deficient mutant *ga-1* do not germinate under any condition, unless GA is supplied exogenously (Koornneef and van der Veen, 1980). Consequently, insensitivity to GA may present a strict measure to inhibit seed germination. Consistent with this hypothesis, reduced germination frequencies were reported for the partially GA-insensitive mutant *Gai* (Koornneef et al., 1985).

In conclusion, the present evidence implies that GA and ABA do not normally interact directly at any stage of seed development. ABA in concert with high ABA-sensitivity appears to be responsible for the induction of dormancy during seed development, and GA in concert with GA-sensitivity induced by dormancy-breaking treatments appears to stimulate germination. Nevertheless, reduction in seed dormancy as a result of low concentrations of ABA (*abe*) or insensitivity to ABA (*abi1, abi2, abi3*) partially relieved the mature seed form a need for GA to induce germination. Seeds of double mutants between *ga-1* and *aba, abi1, abi2* or *abi3*, respectively, were capable of germinating (Karssen et al., 1983; Koornneef et al., 1984, Nambara et al., 1992), whereas *ga-1* single mutants have, thus far, never been shown to germinate without

application of GA (Hilhorst and Karssen, 1992). Since mature seeds contain very low concentrations of ABA, it was not considered likely that the *ge-1* single mutant required GA to directly oppose the action of endogenous ABA present in the seed. In contrast, a "remote control" model was suggested in which the GA requirement for germination depends on the depth of dormancy induced during seed development. Deeply dormant seeds, as wild-type seeds, have a high GA-requirement to promote germination, while seeds with little dormancy (ABA mutants) have a low GA-requirement which may be satisfied by low concentrations of GA present in the possibly leaky *ge-1* mutant.

MATERIALS AND METHODS

Plant Material

Except for immature *d1* mutant kernels, which were obtained from greenhouse-grown plants, all maize developing ears were harvested from field-grown plants. Under the local environmental conditions, kernels typically begin accumulation of anthocyanins at day 17 postpollination and reach seed maturity after ca. 30-33 DAP. The wild-type maize stock used in this study was a color-converted W22 inbred line carrying all factors required for anthocyanin pigmentation of the aleurone. The *vp1-R* allele (Robertson 1955) segregated in a color-converted W22 inbred line carrying all other factors required for anthocyanin pigmentation of the aleurone. This line is routinely maintained by selfing. The *vp1-m2* allele (originally named *vp1-mum2*, McCarty et al., 1989b) arose in *Robertson's Mutator* transposable element stocks (Robertson 1978), but was confirmed to carry an *Mpi* transposable element insertion (D.R. McCarty, unpublished results). Seed segregating for the *vp5* mutation was obtained from the Maize Genetics Corporation Stock Center (University of Illinois, Urbana-Champaign). To produce *vp1,vp5* double mutant seeds, heterozygous *vp5* mutant plants were crossed with heterozygous *vp1-R* mutant plants. A mutation conferring embryo abortion at early globular stage ("germless") arose in a *Robertson's Mutator*-induced mutant screen (D.R. McCarty et al., unpublished results). *Germless* mutant seed were backcrossed into W22 background for at least two generations.

vp1-non-concordant seed was generated using a TB translocation stock (Fig. 2). TB3La seed carrying a BA-translocation on the long arm of chromosome 3, the location of the *Vp1* gene, was obtained from the stock center. This seed contains an extra, normally heterochromatic chromosome, called B-chromosome, in addition to the normal set of A

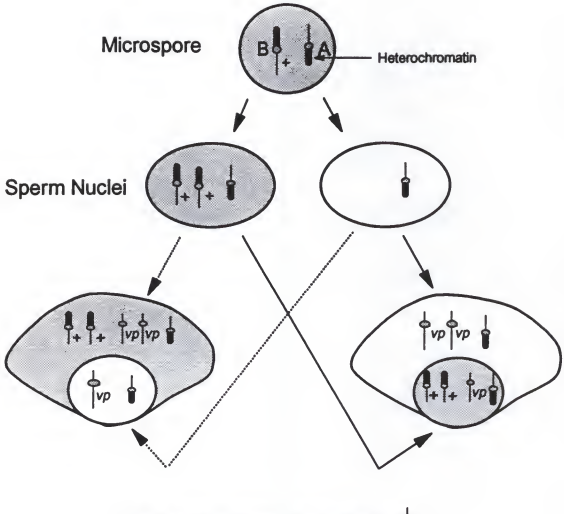


Fig. 2. Generation of *vp1*-non-concordant seed using a TB3La translocation stock.

chromosomes. Due to a translocation event between A-chromosome 3 and the B-chromosome, the 3La part of the A-chromosome 3 is carried by the B-chromosome, while heterochromatic DNA is found on the 3La part of the A-chromosome 3 (Fig. 2). The TB3La stock was crossed to *vp1-R* at least once. Hence, resulting TB3La, $A^{vp1\text{-}R,+}$ B^{+} plants carry one 3A-chromosome segregating for the *vp1* mutation, the homologous 3A chromosome with heterochromatic DNA (thus conferring a *vp1* mutant phenotype) and a B-chromosome carrying the wild-type *Vp1* gene . To obtain *vp1* non-concordant kernels, pollen from TB3La, $A^{vp1\text{-}R,+}$ B^{+} plants is crossed onto segregating *vp1-R* females. During the second pollen mitosis, the replicated B-chromosomes undergo non-disjunction forming one sperm nucleus with two B-chromosomes and one sperm nucleus without a B-chromosome (Fig. 2). Hence, following double fertilization, non-concordant seeds are produced carrying a *vp1* mutant embryo and a wild-type endosperm or vice versa (Fig. 2).

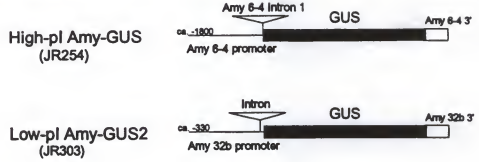
For experiments with germinating wild-type seeds of maize, seeds of the variety NK508 were used (kindly provided by Northrup-King).

Wild-type barley seeds c.v. Himalaya were obtained from Washington State University, Pullman, WA (harvests 1988, 1991 and 1992). Seed segregating for the *slender* mutation (Himalaya background) was kindly provided by P. Chandler. As with seed segregating for *D8*, wild-type and *slender* mutant endosperms were genotyped by germination of the excised embryo.

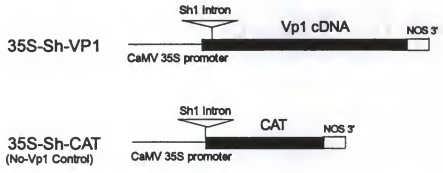
Plasmid Constructs

In all experiments, JR254 (Amy-GUS) or JR303 were used as reporter constructs (see Fig. 3). Amy-GUS and JR303 were kindly provided by J. Rogers and T.H.D. Ho, respectively. Amy-GUS contains ca. 1,800 bp of the 5' flanking sequence of a barley high pI α-amylase gene (*Amy6-4*; Kursheed and Rogers, 1988), the first intron of *Amy6-4*, the GUS reporter gene and the *Amy6-4* 3' terminator. JR303, containing a low-pI α-amylase promoter, was derived from

Reporter Plasmids:



Effector Plasmids:



Internal Standard:

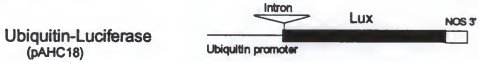


Fig. 3. Plasmid constructs.

Amy32b (Lanahan et al., 1992). For its structure, see Fig 3. As effector construct, 35S-Sh-VP1 was used (McCarty et al., 1991). For no-VP1 control treatments, 35S-Sh-CAT (Vasil et al., 1989) was added instead of 35S-Sh-VP1 to maintain a constant amount of total DNA and 35S promoter in the bombardment mixtures. To normalize for transformation efficiency, a Ubiquitin-Luciferase construct (Ubi-LUC; Bruce et al., 1989) was included into each bombardment mixture. Hence, expression data are presented as Amy-GUS / Ubi-LUC or JR303 / Ubi-LUC ratios. It was confirmed that co-expression of 35S-Sh-VP1 has no effect on expression of Ubi-LUC.

Construction of plasmids carrying an activation domain-deletion derivative of VP1 or a replacement with Herpes simplex virus transcription factor VP16 activation domain was described in McCarty et al. (1991). Internal deletion constructs were made by introduction of two *Nco*I restriction sites and subsequent deletion of the insert and religation of the backbone (Fig. 4). *Nco*I sites were introduced by site-directed mutagenesis using the Altered Sites *in vitro* Mutagenesis System from Promega. Briefly, mutant oligonucleotides and an ampicillin repair oligonucleotide which restores the function of a defective ampicillin resistance gene in the phagemid provided were annealed to single-stranded DNA template. Following DNA synthesis and ligation, the resulting double-stranded phagemid was transformed into a repair-deficient strain of *E.coli* which is subsequently grown in Ampicillin-containing liquid medium for selection. From the obtained bacterial suspension, plasmid DNA was isolated and transformed into an *E. coli* strain conventionally used for transformations (JM83). Colonies growing on Ampicillin were tested for the presence of the desired mutation by restriction enzyme digestion.

The constructs 86/87, 86/85, 85/87, 87/88, 93/95, 103/104, 101/100 and VP1-McW were made available by L. Rosenkrans and D.R. McCarty.

Particle Bombardment and Transient Expression

Tissue preparation

Maize developing ears or dry, mature seeds were surface sterilized in 70% ethanol for 1 min followed by 0.525% NaOCl for 10 min. Dry seeds were germinated in a solution containing

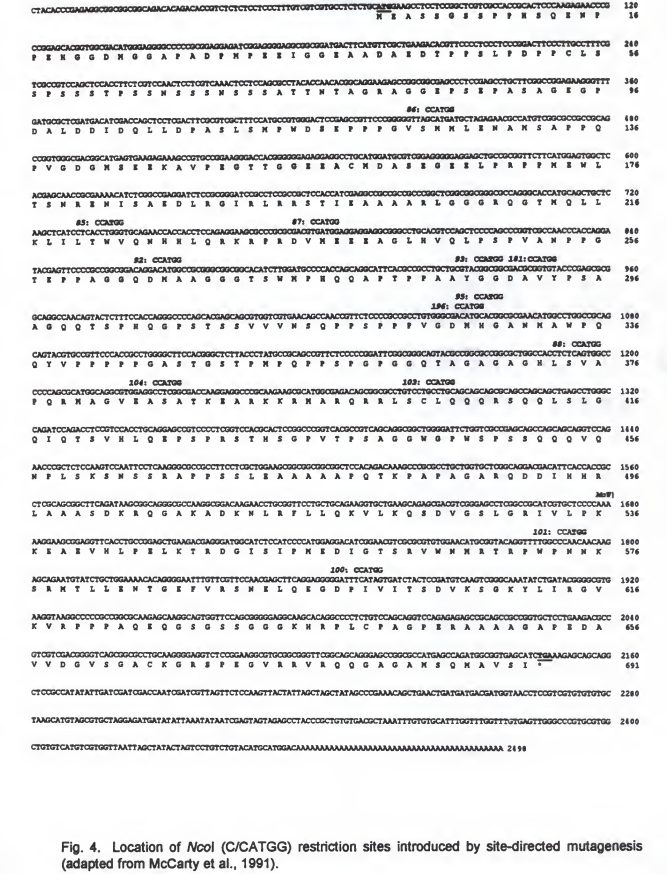


Fig. 4. Location of *NcoI* (C/CATGG) restriction sites introduced by site-directed mutagenesis (adapted from McCarty et al., 1991).

MS salts and MS vitamins (Sigma, cat# M5519) on a gyratory shaker in the dark for ca. 36 hrs, while developing seeds were used immediately. The embryo as well as pericarp and testa tissues were removed from the seeds to expose the aleurone layer of the endosperm. Prepared endosperms were placed on Gelrite-solidified salt medium and then subjected to particle bombardment.

Barley seeds were de-embryonated prior to surface sterilization in 70% ethanol for 1 min followed by 10 min in 1.75 - 2.9 % NaOCl. A minimum of 1.75% NaOCl (=30% Clorox) was necessary to allow easy removal of pericarp and testa layers prior to bombardment. Sterilized half-seeds were imbibed overnight and prepared for particle bombardment as described above for maize seeds.

Particle Bombardment

Particle bombardment was performed as described in Taylor and Vasil (1991) using a DuPont PDS-1000 particle gun. Briefly, 35 to 50 µl of a sterile 50 mg/ml gold stock solution (Biorad, 1.0 or 1.6 µm particle diameter; prepared in water) was mixed with premixed plasmid DNA in a 250 µl-Eppendorf tube and vortexed on maximum speed for 10 s. Immediately, the tube was shifted sideways and 10 µl of 0.1 M spermidine (free base) and 25 µl of 2.5 M $CaCl_2$ were placed onto the side of the tube without allowing it to mix with the gold/DNA solution. Then, the tube was placed upright and subjected to vortexing for 10 s. The precipitated gold/DNA particles were allowed to settle for ca. 3 min, after which part of the supernatant was removed leaving 35-45 µl of liquid behind. The tubes were placed on ice until further use in particle bombardment. For particle bombardment, 2 µl of sonicated gold/DNA solution (containing ca. 80 µg of gold) were used for individual shots.

The bombardment procedure had frequently to be adjusted to the gold characteristics which varied substantially from batch to batch. Modifying the amount of gold used per shot was found most successful in improving bombardment efficiency.

Incubation and Extraction of Endosperms Following Bombardment

Following bombardment, 1 ml of a solution containing MS salts and MS vitamins supplemented with no hormones, 10^{-6} M GA_3 or 10^{-6} M GA_3 and 10^{-4} M (or 10^{-5} M) ABA was dripped over the endosperms. After 24 h of incubation in darkness, maize endosperms were ground either individually (in experiments using developing seeds) or in bulk from each bombardment (when germinating seeds were used) with mortar and pestle aided by the addition of silicon carbide powder in 200-1,000 µl of extraction buffer (0.1 M potassium phosphate (pH 7.8), 2 mM EDTA (pH 8), 2 mM DTT, 5% glycerol). The homogenates were centrifuged to recover the cell extract. To obtain barley aleurone extract, the aleurone layers were separated from the endosperms and ground in bulk for each replicate in 200 µl of extraction buffer. The homogenates had to undergo two rounds of centrifugation to obtain clear cell extract.

Quantification of Transient Expression

Quantitative measurement of GUS activities was performed as described in Jefferson et al. (1987) with the modification that the substrate MUG was dissolved in the extraction buffer described above. For determination of luciferase activities, 10 µl aliquots of the extract and 200 µl of reaction buffer (25 mM tricine (pH 7.8), 15 mM $MgCl_2$, 5 mM ATP, 0.05% BSA) were placed in cuvettes and immediately assayed using a Monolight 2010 luminometer. The luminometer automatically injects 100 µl of 1 mM luciferin and then counts the emitted photons for 15 s. The unit of measurement is the Relative Light Unit (RLU).

RESULTS

Repression of Hydrolase Genes by VP1 in Aleurones of Developing Maize Seeds

Phenotypic analysis of *vp1-m2* kernels

The *vp1-m2* allele of *Vp1* carries a transposon insertion in the third intron which causes somatic instability of the gene during endosperm development (McCarty et al., 1989b). As a result, mosaic kernels develop with clonal *vp1* mutant and wild-type sectors. In these kernels, a striking pattern of endosperm remobilization is often evident. Endosperm tissue underlying *vp1* mutant aleurone cells is frequently softened and depressed in surface while wild-type sectors are raised relative to adjacent mutant sectors. This produces kernels with a distinctive etched appearance (Fig. 5a). The softening response was also observed when only a small fraction of the endosperm was comprised of mutant tissue (Fig. 5b), indicating that expression of this phenotype is cell autonomous. The softening of starchy endosperm tissues that underlie islands of *vp1* mutant aleurone cells appears to be attributed to precocious induction and secretion of hydrolytic enzymes caused by the loss of VP1 function. Thus, repression of hydrolases in developing maize kernels is evidently dependent on the presence of functional VP1.

Transient expression of Amy-GUS in maize aleurone

In order to more directly address the role of VP1 in repressing hydrolase activity, a quantitative transient gene expression assay was developed that is based on particle bombardment of aleurone tissue with a barley high-pI α-amylase promoter-GUS fusion construct (Amy-GUS). It was first interesting to determine whether the observed differential activity of hydrolases in *vp1* mutant and wild-type sectors was due to transcriptional control. For this purpose, Amy-GUS was introduced into aleurone cells of developing *vp1* mutant and

Fig. 5. Cell autonomous de-repression of the aleurone germination response in *vp1-m2* mutant aleurone sectors. (A) The kernel shown is a mosaic: regions pigmented with purple anthocyanin are wild-type; yellow, anthocyanin-deficient regions are clonal sectors of aleurone that have lost *Vp1* function. (B) Magnification of a *vp1-m2* kernel.

wild-type maize seeds. Table 1 shows that during mid-late development Amy-GUS was not expressed in developing wild-type aleurone, even in the presence of exogenous GA. In contrast, GA-induction of Amy-GUS was detected in *vp1* mutant aleurones as early as 20 days after pollination (DAP). These data indicate that in developing wild-type aleurone tissue α-amylase genes are insensitive to GA while in *vp1* mutant aleurone cells α-amylase expression is transcriptionally de-repressed.

Amy-GUS expression in *vp1* mutant aleurone was found to be under developmental and hormonal control. Prior to approx. 18 DAP, Amy-GUS was inactive in GA-treated as well as untreated aleurone, indicating that early in seed development the aleurone is unresponsive to GA even in the absence of VP1 protein. At 20 DAP, Amy-GUS was induced by exogenous GA, whereas its activity remained low in untreated aleurone (Fig. 6). Late in seed development (24 DAP), Amy-GUS was constitutively active in the absence of GA, indicating a greatly reduced dependence on exogenous hormone. Two observations, however, indicate that Amy-GUS expression was not fully constitutive at this stage: 1) GA treatment significantly enhanced AMY-GUS expression (as much as 3-fold over that of untreated aleurones) in some, but not all experiments (Table 1, Fig. 6). 2) GA treated aleurones typically exhibited less quantitative variation in Amy-GUS expression than non-treated sibling aleurones. The latter effect suggests that developmental or spatial variation affecting endogenous hormone concentrations within the ear or seed might contribute to the large variation observed in the absence of exogenous GA.

The differential expression of Amy-GUS in developing *vp1* mutant and wild-type aleurone cells confirms a role of VP1 in the repression of α-amylase genes during seed development. In order to test whether expression of recombinant VP1 could evoke inhibition of α-amylase transcription in *vp1* mutant aleurones, aleurones were bombarded with a mixture of Amy-GUS and 35S-Sh-VP1 plasmids. Co-expression of VP1 strongly inhibited Amy-GUS expression in *vp1* mutant aleurone in the presence as well as absence of exogenous GA (Fig. 6), indicating that recombinant VP1 effectively restored the wild-type phenotype. We can rule out the possibility that over-expression of VP1 causes non-specific squelching of general

	Amy-GUS / LUC *10^4 [pmoles MU/h/RLU]							
	vp1-R mutant Aleurones						Wild-type Aleurones	
Days after	-GA			+GA			-GA	+GA
Pollination	Range	Mean	S.E.M.	Range	Mean	S.E.M.	Mean	Mean
18		<1			<1		<1	<1
20		<1		4-35	21	±7	<1	<1
24	114-531	263	±130	41-150	110	±35	<1	<1

Table 1. Amy-GUS is inducible in *vp1-R* mutant aleurone cells but not in wild-type aleurone cells. Aleurones of developing *vp1-R* mutant and wild-type kernels at 18, 20 and 24 DAP were bombarded with a mixture of 10 µg of Amy-GUS and 5 µg of Ubi-LUC. Post-bombardment, kernels were treated with a solution containing no hormones or 10^{-6} M GA_3. Data represent mean (± S.E.M) of three to five replicates.

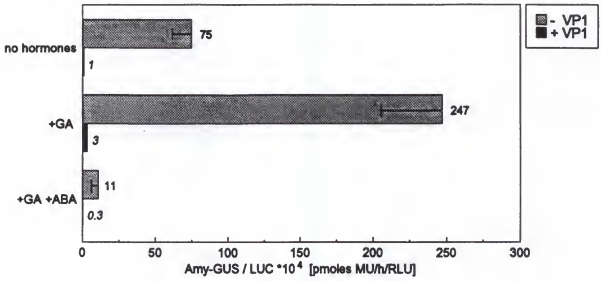


Fig. 6. Effect of VP1 over-expression and ABA on Amy-GUS expression in *vp1-R* mutant aleurone. *vp1-R* mutant aleurones from kernels harvested 26 DAP were bombarded with 10 μg of Amy-GUS, 5 μg of Ubi-LUC, and 5 μg of 35S-Sh-VP1 or 35S-Sh-CAT (for no-VP1 controls). Following bombardment, a solution containing no hormones, 10^{-6} M GA_3 or 10^{-6} M GA_3 and 10^{-4} M ABA was applied to the kernels. Numbers behind bars represent means of five replicates. Error bars show S.E.M.

transcription factors because no inhibitory effect of co-bombarded VP1 on 35S-Sh-GUS or Ubiquitin-Luciferase expression was observed (data not shown). Moreover, VP1 caused *trans*-activation of positively regulated reporter constructs, Em-GUS and C1-Sh-GUS, in aleurone cells using similar bombardment conditions (S. Cocciolone and D.R. McCarty, unpublished results).

Interaction between VP1 and Abscisic Acid

In concert with VP1, the hormone ABA plays an important role during seed maturation (McCarty and Carson, 1991). Moreover, ABA functions as an inhibitor of α-amylase expression in germinating cereal seeds (Jacobsen and Chandler, 1987). This suggests that ABA might also be involved in repression of α-amylase genes in the developing seed. Therefore, possible interactions between ABA and VP1 in repressing Amy-GUS were analyzed.

ABA was effective in blocking Amy-GUS expression in *vp1* mutant aleurone (Fig. 6). This indicates that repression by ABA does not require VP1. In combination, ABA and VP1 over-expression produced a roughly additive effect (Fig. 6).

To test whether α-amylase repression by VP1 is dependent on ABA, recombinant VP1 was over-expressed in aleurone of developing *vp1,vp5* double mutant kernels that are deficient for ABA biosynthesis. Figure 7 shows that VP1 was highly effective in repressing Amy-GUS in *vp5* mutant background. While it cannot by ruled out that maternal ABA derived from the *vp5/+* parent plant may be sufficient for VP1 function, it is suggested that VP1-mediated repression of Amy-GUS expression does not require ABA. This would be consistent with the finding that VP1 also functions in aleurone of germinating seeds (see below) where ABA levels are very low (Oishi and Bewley, 1990). Taken together, these data suggest that ABA and VP1 inhibit Amy-GUS expression independently.

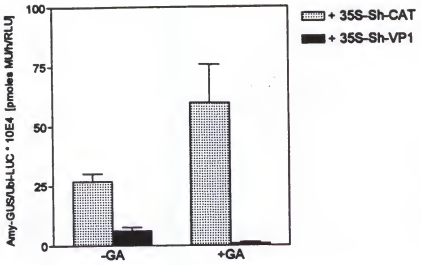


Fig. 7. Co-expressed VP1 inhibited Amy-GUS in aleurone of developing *vp1/vp5* double mutant seeds that are deficient for ABA biosynthesis. Kernels were harvested 24 DAP. Bombardments were performed as described in Fig. 6. Following bombardment, kernels were incubated in no hormones or 10^{-6} M GA_3. Data represent mean (± S.E.M) of 7-8 replicates.

Effector Construct	Amylase-GUS / Ubi-LUC * 10^4 (±S.E.M.) [pmoles MU/hr/RLU]			
	Maize Seeds		Barley Seeds	
	Amy-GUS	JR303	Amy-GUS	JR303
35S-Sh-CAT (Control)	91 (±17)	1.19 (±0.35)	247 (±36)	65 (±5.2)
35S-Sh-Vp1	10.5 (±0.6)	0.07 (±0.014)	70 (±16)	25 (±6.4)

Fig. 8. Co-expressed VP1 inhibited Amy-GUS and JR303 in aleurone of germinating maize and barley seeds. Aleurones of imbibed seeds were bombarded with Amy-GUS (maize: 4 µg; barley: 2 µg) or JR303 (maize: 10 µg; barley: 5 µg), 5 µg of Ubi-LUC, and 5 µg of 35S-Sh-VP1 or 35S-Sh-CAT. Post-bombardment, kernels were incubated in 10^{-6} M GA_3. Data represent mean (± S.E.M) of 3-5 replicates.

Over-expression of VP1 in Aleurones of Germinating Maize and Barley Seeds

Endogenous expression of *Vp1* in embryo and aleurone tissues is under strict developmental control. *Vp1* mRNA peaks at 16 DAP and then gradually decreases as the seed reaches maturity (McCarty et al., 1991). Germinating seeds, in contrast, display no *Vp1* expression or detectable levels of VP1 protein (Carson, 1992). Thus, VP1 function in maize is limited to the maturing seed. To test whether VP1 can function in germinating seeds in a way equivalent to maturing seeds, we co-expressed 35S-Sh-VP1 and Amy-GUS in aleurones of germinating wild-type seeds of maize. In the presence of exogenous GA, VP1 reduced Amy-GUS expression by ca. 90% (Fig. 8). Thus, VP1 also functions in germinating seeds, apparently without the need for additional developmental factors. Furthermore, VP1 also repressed expression directed by the barley low-pi α-amylase promoter (JR303) which shows considerable sequence divergence from Amy-GUS. This indicates that expression of high- as well as low-pi α -amylase genes is under control of VP1.

Because germination-specific responses are well characterized in barley, VP1-mediated repression was tested in aleurones of germinating barley seeds. Though not as effective as in maize, VP1 also inhibited Amy-GUS and JR303 expression in barley (Fig. 8).

Moreover, variation within as well as between experiments was significantly reduced in aleurones of germinating wild-type seeds of maize and barley as compared to aleurones of developing *vp1* mutant maize seeds. Therefore bombardment of germinating seeds constitutes a useful experimental system to further characterize VP1 function.

A VP1 dose-response curve was generated to determine the amount of co-expressed VP1 necessary to achieve maximum repression of Amy-GUS in barley aleurone cells. Figure 9 shows that repression was already evident when 1.25 µg of 35S-Sh-VP1 were co-transferred with Amy-GUS, while increasing the amount of 35S-Sh-VP1 beyond 2.5 µg did not lead to further repression of Amy-GUS. Hence, comparatively low amonts of recombinant VP1 are sufficient to achieve inhibition of Amy-GUS. To confirm that the inhibitory effect of VP1 on Amy-GUS in barley aleurone is promoter-specific, 35S-Sh-VP1 was co-expressed with an Em-GUS reporter

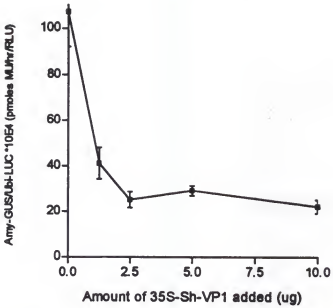


Fig. 9: VP1 dose-response for repression of Amy-GUS in aleurone of germinating barley seeds. 0 to 10 µg of 35S-Sh-VP1 were co-precipitated with 0.5 µg of Amy-GUS and 5 µg of Ubi-LUC. Total amount of DNA was balanced by addition of 35S-Sh-CAT. Post-bombardment, endosperms were cultured in 10^{-6} M GA_3. Data represent mean (± S.E.M) of five replicates.

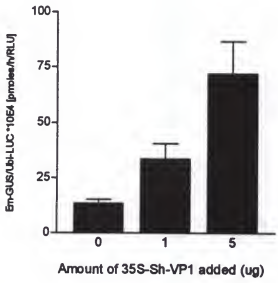


Fig. 10: Co-expression of VP1 activated Em-GUS in barley aleurone. Aleurones of germinating barley seeds were bombarded with 2 µg of Em-GUS, 5 µg of Ubi-LUC and 0, 1 or 5 µg of 35S-Sh-VP1. Total amount of DNA was balanced by addition of 35S-Sh-CAT. Post-bombardment, endosperms were cultured in no hormones. Data represent mean (± S.E.M) of five replicates.

construct containing the full length promoter of the wheat *Em* gene fused to the GUS coding sequence (Marcotte et al., 1989). Recombinant VP1 increased expression of Em-GUS by ca. 5-fold (Fig. 10) which is consistent with the function of VP1 as a transcriptional activator of *Em* (McCarty et al., 1991). In conclusion, these data show that VP1 can repress or activate gene transcription depending on the promoter context.

Interaction between VP1 and Gibberellic Acid

The well characterized hormonal responses in Himalaya barley aleurone facilitated further studies regarding the interaction between VP1 and GA. For this purpose, GA response curves of Amy-GUS expression were determined in aleurones of de-germed imbibed Himalaya "half seeds" (Fig. 11). In the absence of co-expressed VP1, Amy-GUS expression showed a typical GA-induction. In contrast, when a mixture of Amy-GUS and recombinant VP1 was introduced into aleurone cells, GA-induction of Amy-GUS expression was reduced by ca. 80%. Most noticeably, the clearly detectable basal activity of Amy-GUS was not significantly affected by co-expression of VP1 (Fig. 11, insert). Thus, VP1 only inhibited the GA-dependent activity of the α-amylase promoter. This implies that VP1 may interfere with the GA signalling pathway.

Recessive mutations that cause constitutive GA-response have been identified in barley and a few other species (Ross, 1994). Barley *slender* (*sln*) mutant plants are characterized by excessive elongation of stem and leaf tissues and constitutive expression of hydrolytic enzymes in the aleurone of imbibed half seeds in the absence of exogenous GA. The mutant phenotype suggests that the *Sln* gene encodes a negative regulator that is normally inactivated by GA (Chandler, 1988; Lanahan and Ho, 1988). To test whether VP1 inhibitory function depends on the presence of the SLN protein, aleurones of *sln* mutant half seeds were co-bombarded with Amy-GUS and recombinant VP1. Figure 12 shows that VP1-mediated repression of Amy-GUS was as effective in *sln* mutant aleurones as in wild-type aleurones.

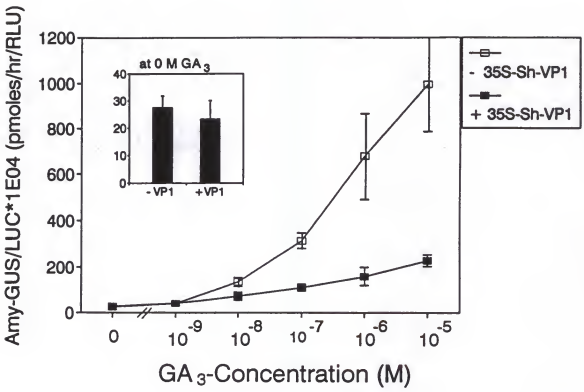


Fig. 11. Co-expression of VP1 inhibited GA_3-induction of Amy-GUS but did not affect its basal activity in aleurone of germinating barley half seeds . Aleurones were bombarded with 2 μg of Amy-GUS, 5 μg of Ubi-LUC, and 5 μg of 35S-Sh-VP1 or 35S-Sh-CAT. Following bombardment, 3 replicates of 5 kernels each were incubated in 0-10^{-5} M GA_3. Data represent mean of three replicates (± S.E.M). The insert shows activities in the absence of GA_3.

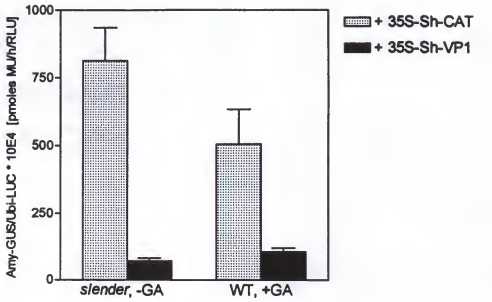


Fig. 12. VP1 function does not require the *Slender* gene product. Aleurones of imbibed *slender* (*sln*) mutant and wild-type barley half seeds harvested from a plant segregating for the *sln* mutation were bombarded with 5 μg of Amy-GUS, 5 μg of Ubi-LUC, and 5 μg of 35S-Sh-VP1 or 35S-Sh-CAT. Following bombardment, *sln* mutant or wild-type seeds were incubated in a solution containing no hormones or 10^{-6} M GA_3, respectively. Data represent mean (± S.E.M.) of 10 replicates.

This indicates that VP1 is likely to act further downstream in, or independently of, the SLN pathway.

While GA is a strong inducer of α-amylase genes in aleurones of Himalaya barley and other cereals, the importance of GA in the regulation of maize α-amylase genes is less clear. α-amylase activities were found high in isolated endosperms that had been de-embryonated prior to imbibition (Harvey and Oaks 1974; Goldstein and Jennings, 1978). Moreover, application of exogenous GA to isolated endosperms did not further enhance α-amylase activities (Oishi and Black, 1990). Hence, it was argued that mature endosperms store high concentrations of GA (Harvey and Oaks 1974; Goldstein and Jennings, 1978; Oishi and Black, 1990). However, seeds of the GA-deficient, extremely dwarfed mutant *d5* displayed considerable α-amylase activity that was only 3-fold lower than in wild-type seeds, implying a GA-independent component in maize α-amylase production. To investigate this, Amy-GUS was introduced into aleurones of germinating wild-type and GA_1-deficient *d1* mutant seeds. Both genotypes displayed similar, high Amy-GUS activities in the absence of exogenous GA (Figs.13, 14). Furthermore, application of GA to *d1* mutant seeds increased Amy-GUS expression by less than two-fold, thus to a similar extent as in wild-type seeds (Figs.13, 14). Hence, the *d1* mutation did not appear to alter Amy-GUS expression in the aleurone, suggesting that deficiency in the highly active gibberellin GA_1 does not severely affect high-level expression of α-amylase genes. Thus, consistent with the data on the mutant *d5* (Harvey and Oaks, 1974), the possibility of a constitutive activity of α-amylase genes in the absence of GA appears likely. The finding that co-expression of VP1 and application of ABA reduced Amy-GUS activity to a very low level (Figs. 13, 14) indicates that VP1 and ABA repress the GA-dependent as well as the putative constitutive activity of Amy-GUS in maize.

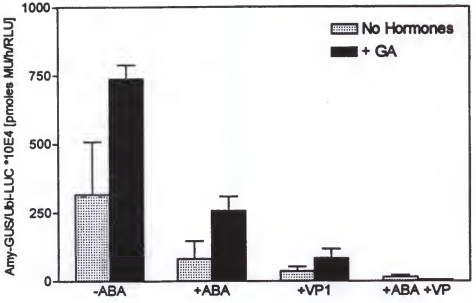


Fig. 13. Effect of GA, ABA and recombinant VP1 on Amy-GUS expression in aleurone of germinating wild-type (NK508) seeds. Aleurones were bombarded with 4 µg Amy-GUS, 5 µg of Ubi-LUC and 5 µg of 35S-Sh-VP1 or 35S-Sh-CAT and then incubated in a solution containing no hormones, 10^{-6} M GA_3, 10^{-5} M ABA, or 10^{-6} M GA_3 and 10^{-5} M ABA. Data represent mean (± S.E.M.) of five replicates.

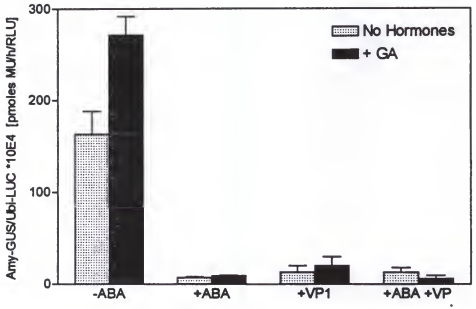


Fig. 14. Effect of GA, ABA and recombinant VP1 on Amy-GUS expression in aleurone of germinating *d1* mutant seeds. Methods as in Fig. 13.

Role of the Embryo in Repression of α-Amylase Genes in the Aleurone

The differential response of mutant and wild-type aleurone tissue in developing *vp1-m2* kernels (Fig. 5a,b) did not appear to be fully independent of the state of the embryo. Embryos of *vp1-m2* seed are frequently non-viviparous and survive desiccation. It was observed that mosaic aleurones that were associated with non-viviparous embryos very rarely exhibited precocious endosperm remobilization while those with viviparous embryos typically did. Moreover, aleurone near the germinal face and crown of the kernel was more strongly affected than aleurone on the abgerminal face. This phenotype suggests that signalling from the embryo as well as responsiveness of the aleurone cells contribute to the softening response.

In order to assess the impact of the physiological state of the embryo on α-amylase expression in aleurone cells, TB3La translocation stocks were used which allow the generation of *vp1* non-concordant seeds with embryo and endosperm of different genetic constitution (for a brief description of the system see Materials and Methods). As a result, ears developed that segregated four genotypes: 1) seeds with a *vp1* mutant embryo and a wild-type endosperm, 2) seeds with a wild-type embryo and a *vp1* mutant endosperm, 3) concordant *vp1* mutant seeds and 4) concordant wild-type seeds. Aleurones of these four genotype combinations were bombarded with Amy-GUS and postbombardment cultured in the absence of added hormones. Amy-GUS was only expressed in aleurones of concordant *vp1* mutant kernels and not in any of the other three genotype combinations (Fig. 15). These data are consistent with the observation made in *vp1-m2* seeds that endosperm tissue underlying *vp1* mutant aleurone was remobilized predominantly in viviparous seeds. Thus, α-amylase genes appear to be de-repressed in aleurone cells that lack functional VP1 predominantly if the embryo is also viviparous. In some experiments in which kernels very late in development were used, both *vp1* non-concordant genotypes displayed significant Amy-GUS activities, while no Amy-GUS expression was detected in aleurones of concordant wild-type seeds (data not shown). This indicates that partial de-repression of Amy-GUS in aleurone cells is facilitated if either the embryo is viviparous or the aleurone cells lack functional VP1.

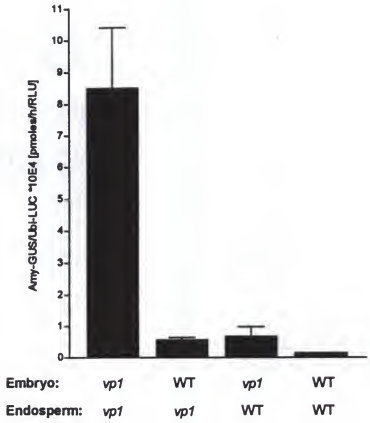


Fig. 15. Amy-GUS expression in aleurone of developing *vp1* non-concordant maize seeds. Aleurones (31 DAP, fall season) were bombarded with 10 µg of Amy-GUS and 5 µg of Ubi-LUC and cultured post-bombardment in no hormones. Data represent mean (± S.E.M.) of six replicates.

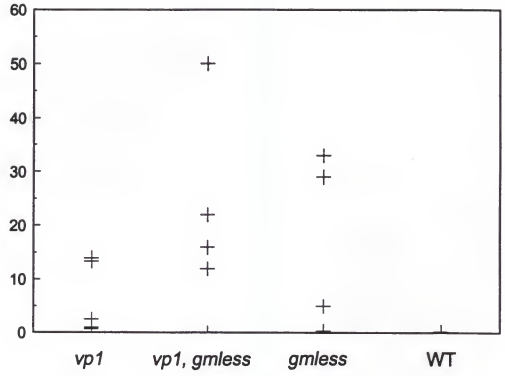


Fig. 16. Amy-GUS expression in aleurone of developing germless seeds. Aleurones (29 DAP, fall season) of an ear segregating for the mutations *vp1* and *germless* were bombarded and cultured as described in Fig 15. Crosses represent single data points of six replicates (exception: four replicates in the *vp1/germless* double mutant).

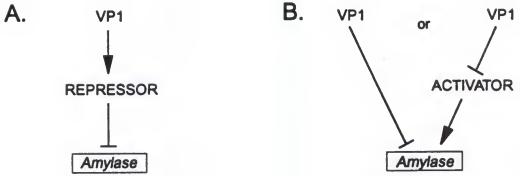
There are at least two possible scenarios that would explain how the state of the embryo might influence de-repression of α-amylase genes in *vp1* mutant aleurone: 1) a viviparous embryo might secrete an inductive signal required for α-amylase expression in the aleurone of developing seeds (e.g. GA), and/or 2) a non-viviparous embryo might contribute a diffusible inhibitory signal that prevents α-amylase expression in the aleurone (e.g. ABA). In order to test these hypotheses, Amy-GUS was introduced into aleurone cells of a germless mutant in which the embryo aborts during the early globular state (P. Becraft and D.R. McCarty, pers. communication). Hence, use of ears that segregate for the *germless* and *vp1* mutations allows assessment of aleurone responsiveness in the absence of a signal from the embryo. Amy-GUS was highly de-repressed in *vp1*-mutant aleurone of germless seeds. Aleurones of all four seeds bombarded expressed Amy-GUS (Fig. 16). This indicates that a viviparous embryo per se is not required for de-repression of α-amylase genes in *vp1* mutant aleurone cells. Rather, it appears that the lack of a normal (non-viviparous) embryo caused induction of Amy-GUS in the *germless,vp1* double mutant. This suggests that a wild-type embryo secretes a signal with inhibitory function on α-amylase expression in the aleurone.

In the single mutants *vp1* and *germless*, Amy-GUS was partially de-repressed (2-3 seeds of a total of six bombarded expressed Amy-GUS, Fig. 16). Only wild-type seeds displayed complete repression of Amy-GUS (Fig. 16). Hence, both VP1 expression in the aleurone and a normal embryo appear to be required for complete inhibition of α-amylase genes in the aleurone. This is consistent with the Amy-GUS activities found in *vp1* non-concordant kernels very late in development, as described above.

Functional Analysis of the VP1 Protein

The acidic activation domain

We considered two models of how VP1 may function in repression of the aleurone germination response. 1) VP1 might be a transcriptional activator of an intermediate repressor gene that in turn inhibits expression of α-amylase genes (Figure 17a). 2) VP1 itself might



C.

Effector Construct		Rel. Amy-GUS / LUC	
		Maize	Barley
35S-Sh-CAT (Control)		100 (±25)	100 (±17)
WT-VP1	activator	16 (±2)	42 (±8)
△28-121		13 (±4)	17 (±8)
3x (VP16 act)		n.d.	12 (±7)

Fig. 17. Mode of action of VP1 in repressing Amy-GUS.
(A),(B). Alternative models for VP1 action as described in the text.
(C). Effect of deletion and substitution derivatives of VP1 on Amy GUS expression in aleurone tissue of germinating maize and barley seeds cultured in GA_3. In △28-121, the activation domain of VP1 was deleted. In 3x (VP16 act), the activation domain of VP1 was replaced by three copies of the Herpes simplex VP16 activation domain. Data represent activities (mean ± S.E.M) relative to control (=100). Black boxes show sequence homology between VP1 and ABI3. (n.d.: not determined).

function as a repressor of the α-amylase genes or of an intermediate gene that is required for activation of the α-amylase promoter (Figure 17b). In order to distinguish between these models, we determined whether the transcriptional activation domain of VP1 which is essential for activation of the *Em* and *C1* genes in maize cells is also required for inhibition of α-amylase. Figure 17c shows that a deletion derivative of VP1 that lacks the N-terminal activation domain was as effective in repressing Amy-GUS expression in maize and barley as the full-length protein. In addition, a VP16/VP1 hybrid protein that contains three copies of the VP16 acidic activation domain and has a restored capacity to activate Em-GUS and C1-Sh-GUS (McCarty et al., 1991; Rosenkrans and McCarty, unpublished results) was not more effective than the activator deletion mutant in causing repression of Amy-GUS. The lack of a requirement for the activation sequence clearly distinguishes the mechanism of VP1-mediated repression from the mechanism of activation of diverse maturation related genes by VP1. These results strongly indicate that the VP1 protein has a discrete repressor function.

Identification of Sequences Essential for the Repressor Function of VP1

A number of internal deletion constructs were tested for their ability to repress Amy-GUS in maize and barley aleurone (Fig. 18). A large ca. 350 bp deletion (86/87) entirely abolished VP1 repressor function, indicating that the deletion-derivative may lack a functionally important domain, or the deletion may affect the spacing and thereby the function of domains present outside this sequence. An only slightly smaller deletion in this region (86/85) did not affect repression in maize nor barley aleurone, suggesting that altered spacing is not likely the reason for the failure of 86/87 to repress. Indeed, a small deletion of 42 bp in the C-terminal half of 86/87 (85/87) abolished repression in barley and consistently reduced activity in maize aleurone by ca. 50%. Hence, this region (hereafter referred to as the RED domain) appears to be essential for VP1 repressor function. Consistent with this conclusion, a large part of the sequence of the RED domain (W V Q N H^+ H^+ L Q R^+ K^+ R^+ P R^+ D^-) is highly charged, predicting this domain to be positioned on the surface of the folded VP1 protein, thus accessible for interactions with other molecules.

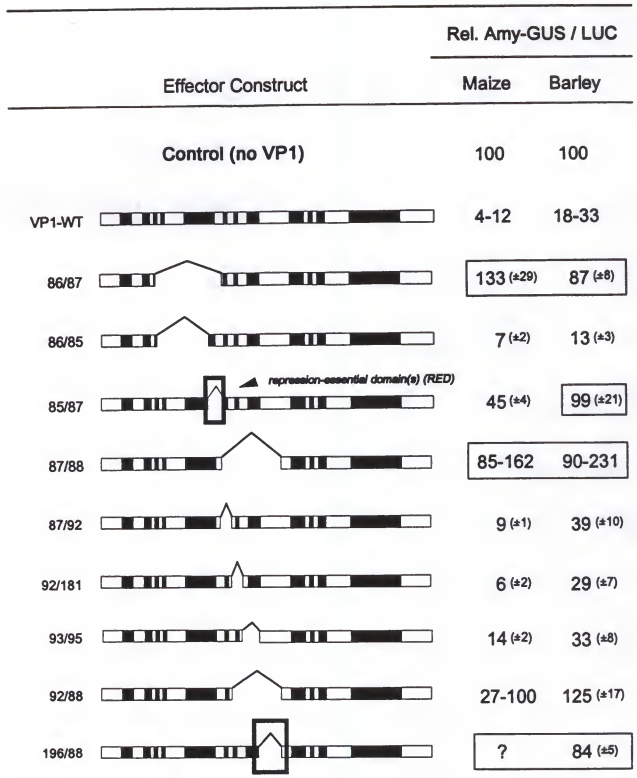


Fig. 18. Deletion analysis of the VP1 protein: Part I. For Materials and Methods see Fig. 19. Black boxes indicate sequence homology to the VP1 homolog from barley.

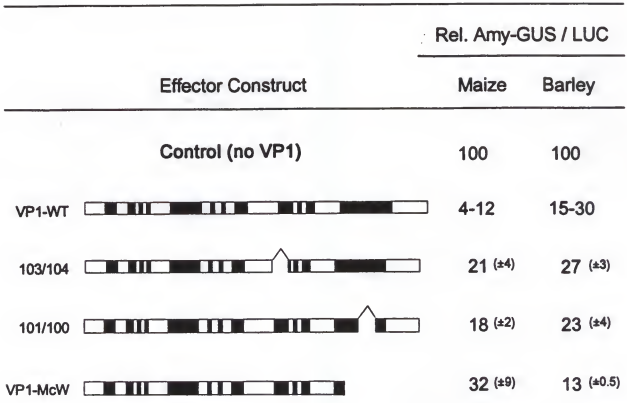


Fig. 19. Deletion analysis of the VP1 protein: Part II. Aleurones of maize and barley germinating seeds were bombarded with 2-5 µg of Amy-GUS, 5 µg of Ubi-LUC and 5 µg of effector construct and then cultured in 10^{-6} M GA_3. Data represent mean (± S.E.M.) of 3-5 replicates

A second large, ca. 400 bp deletion (87/88) also rendered the VP1 protein incapable of repressing Amy-GUS (Fig. 18). The activity of this construct varied to an unusual extent, from slight, but non-significant repression in some experiments to more than two-fold, statistically significant activation of Amy-GUS in others. Similarly, a construct with a slightly smaller deletion of this region (92/88) displayed a highly variable effect. Subsequently, four adjacent sub-deletions within the 87/88 domain were constructed. Each of the N-terminal three deletions (87/92, 92/181, 93/95) eliminates one region that is conserved in the barley VP1 homolog, but none of these deletions severely diminished repression. In contrast, the C-terminal, ca. 140 bp deletion 196/88 eliminating a non-conserved stretch of VP1 almost entirely abolished repressor function, implying that this region may contain an important site involved in Amy-GUS repression.

Deletions in the C-terminal portion of VP1 did not strongly affect repression of Amy-GUS (Fig. 19). Truncation of the C-terminal 450 bp (VP1-McW) – generating the product of the *vp1-McWhirter* allele which confers a non-viviparous, anthocyanin-deficient phenotype – had only a slight effect in maize, while not affecting repression in barley. Similarly, deletion of the domains 101/100 and 103/104 did not strongly diminish Amy-GUS repression.

The 87/88 deletion mutant

It was shown that in the absence of GA, co-expression of VP1 had no effect on Amy-GUS expression in aleurone of germinating barley half seeds (see Fig. 11). In contrast, when the 87/88 deletion-derivative of VP1 was over-expressed with Amy-GUS in the absence of GA, Amy-GUS expression was activated (Fig. 20). This activation was highly variable, ranging from 2-fold in some experiments to up to 12-fold in others. It appeared specific to the 87/88 deletion mutant and was not found for any other tested constructs containing deletions outside this region of VP1 (data not shown). Interestingly, activation of Amy-GUS by 87/88 was also observed in the presence of ABA (Fig. 21). In the presence of GA, a slight activation of Amy-GUS (max. 2-fold) by 87/88 was observed in some, but not all experiments (Figs. 18, 20).

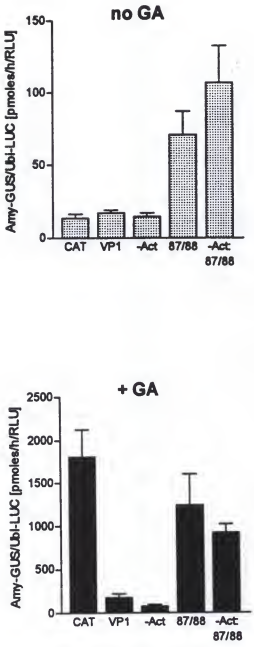


Fig. 20. The VP1 deletion-derivative 87/88 activates Amy-GUS in the absence of GA in aleurones of germinating barley seeds. Aleurones were bombarded with 2 µg of Amy-GUS, 5 µg of Ubi-LUC and 10 µg of either 35S-Sh-CAT, 35S-Sh-VP1, the activation domain-deletion mutant described in Fig. 18 (-Act), 87/88 or the double mutant that carries deletions of the activation domain and 87/88 (-Act:87/88), respectively. After bombardment, endosperms were cultured in no hormones (top graph) or 10^{-6} M GA_3 (bottom graph). Data represent mean (± S.E.M.) of three replicates.

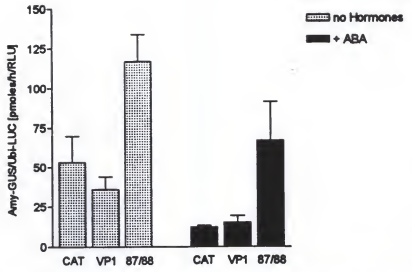


Fig. 21. ABA does not inhibit 87/88-mediated activation of Amy-GUS found in the absence of GA. Aleurones of germinating barley seeds were bombarded as described in Fig. 20 and then cultured in no hormones or 10^{-5} M ABA.

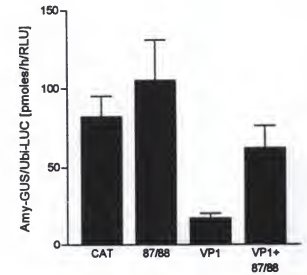


Fig. 22. The VP1 deletion mutant 87/88 displays a dominant negative effect on VP1-mediated repression of Amy-GUS. Aleurones of germinating barley seeds were bombarded with 6 µg of Amy-GUS, 5 µg of Ubi-LUC and either 25 µg of CAT, 20 µg of 87/88, 5 µg of VP1 or both 20 µg of 87/88 and 5 µg of VP1. To all mixtures, CAT plasmid was added to obtain a total amount of 36 µg of plasmid DNA. After bombardment, endosperms were cultured in 10^{-6} M GA_3. Data represent mean (± S.E.M.) of five replicates.

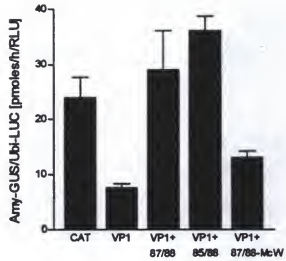


Fig. 23. Effect of the double deletion mutants 85/88 and 87/88:McW on inhibition of Amy-GUS by over-expressed VP1. Materials and Methods as described in Fig. 22. The double mutants 85/88 and 87/88:McW were constructed by restriction enzyme digestion and subsequent ligation. Data represent mean (± S.E.M.) of 5-6 replicates.

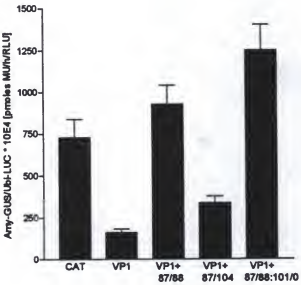


Fig. 24. Effect of the double deletion mutants 87/104 and 87/88:101/100 on inhibition of Amy-GUS by over-expressed VP1. Materials and Methods as described in Fig. 22.

The finding that 87/88 activates Amy-GUS indicates that the mutant protein is not fully non-functional. The data suggest that 87/88 may be capable of interacting with a normal component of the repression mechanism but unable to cause repression. In doing so, there are at least two possibilities as to how it might activate Amy-GUS. 1) The acidic activation domain of 87/88 might elicit transcriptional activation of α-amylase genes or an intermediate gene. In the wild-type VP1 protein, this activity might either be not accessible or masked by the repressor function. 2) In producing a non-functional complex, 87/88 might compete with, or titrate out, an endogenous repressor (e.g. endogenous barley VP1-homolog possibly present in aleurone of germinating barley seeds) and thus exert a dominant negative effect.

In order to test the first possibility, a double deletion mutant was constructed that deletes the 87/88 domain and the acidic activation sequence. This double mutant was as effective in activating Amy-GUS as the 87/88 single mutant (Fig. 20), suggesting that the transcriptional activation domain is not involved. Therefore, it was tested whether 87/88 is capable of inhibiting the effect of recombinant VP1. 87/88 and recombinant VP1 were expressed by themselves and in combination (ratio 4:1) together with Amy-GUS in barley aleurone. Co-expression of 87/88 reduced VP1-mediated repression of Amy-GUS by ca. 75% (Fig. 22). This is consistent with the view of a dominant negative effect of 87/88.

To identify domains involved in mediating the dominant negative effect of 87/88, double-deletion mutants deleting 87/88 and other sequences of the VP1 protein were constructed and tested for their ability to reduce repression of Amy-GUS by co-expressed VP1. Double mutants deleting the domains 87/88 and 85/87 (the RED domain) or 101/100, respectively, were as effective in competing with recombinant VP1 as the 87/88 single mutant (Figs. 23, 24). In contrast, the double mutants deleting 87/88 and either the C-terminal 450 bp of VP1 (87/88:McW) or the domain 103/104 (87/104) did not show a dominant negative effect on Amy-GUS repression by co-expressed VP1 (Figs. 23, 24), suggesting that these domains may be important for the inhibitory role of 87/88 on VP1 repressor function.

DISCUSSION

VP1 of maize is a transcription factor that is specifically expressed in the developing seed (McCarty et al., 1989a, 1991). It was shown previously that VP1 is required for ABA-induced activation of a variety of genes associated with seed maturation (McCarty et al., 1991). Results of this work show that, in addition to its transcriptional activator function, VP1 has a specific role in blocking precocious induction of germination-specific α-amylase genes during seed development.

VP1 Represses α-Amylase Genes

This study provides at least three lines of evidence that indicate a function of VP1 in repression of α-amylase genes in the developing seed. First, somatically unstable *vp1-m2* seeds containing both *vp1* mutant and wild-type sectors displayed cell autonomous de-repression of endosperm remobilization specifically in sectors underlying *vp1* mutant aleurone (Fig. 5a,b). Second, in transient expression experiments Amy-GUS was inducible or constitutively active in developing *vp1* mutant aleurone cells but not in wild-type aleurone cells (Table 1). Third, co-expression of recombinant VP1 with Amy-GUS in *vp1* mutant aleurone cells inhibited Amy-GUS expression by >95% (Fig. 6). These results are consistent with findings that α-amylase genes are not expressed in the developing seed (Nicholls, 1979; Comford et al., 1986; Garcia-Maya et al., 1990; Oishi and Bewley, 1990). Hence, cessation of VP1 expression prior to germination may be necessary to allow induction of α-amylase genes in the germinating seed.

Gene Repression is a Discrete Function of VP1

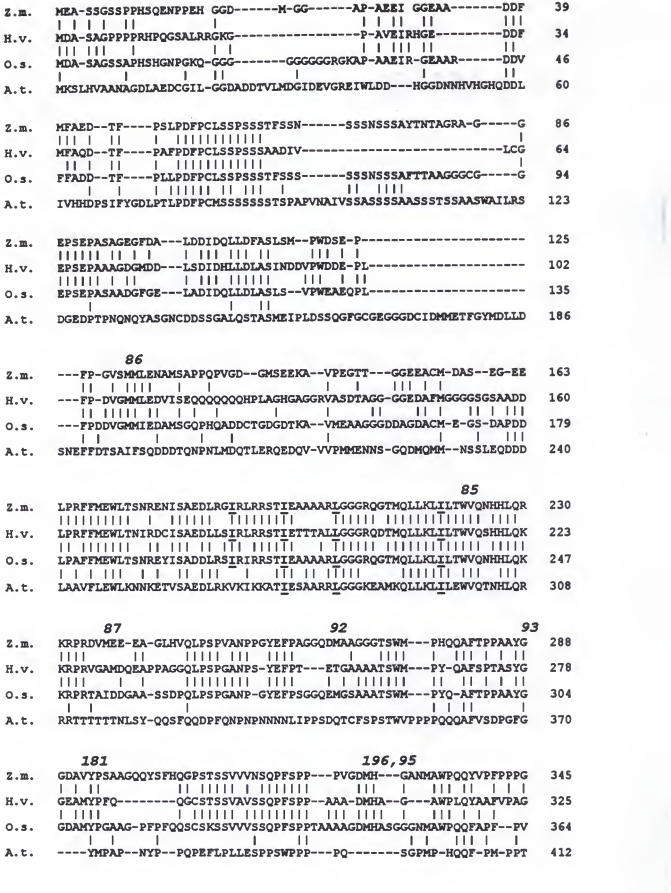
In contrast to the mechanism of transcriptional activation of maturation-specific genes, VP1-mediated repression of α-amylase genes does not require the transcriptional activation function located at the N-terminal domain of VP1 (Fig. 17). This indicates that VP1 has a discrete repressor function that is mechanistically distinct from the transcriptional activation function. Several systems in which a single transcription factor functions as both an activator and a repressor depending on the target promoter have been described in animals (Miner and Yamamoto, 1991; Tsai and O'Malley, 1994). Direct structural homologs of VP1 are thus far known only in plants, suggesting that this strategy has evolved independently in plants and animals.

Functional Analysis of the VP1 Protein

To identify domains in the VP1 protein that are important for repressor function, mutant derivatives containing deletions covering ca. 80 % of the total protein were tested for their ability to inhibit Amy-GUS. Deletion of very highly conserved sequences in the C-terminal half of VP1 (103/104, 101/100, McW) did not, or only slightly, reduce repressor function (Fig. 19). In contrast, two constructs deleting sequences in the middle of the VP1 protein (85/87, 87/88) were strongly affected in repression of Amy-GUS (Figs. 18). While disruption of VP1 function is one possibility for lack of Amy-GUS repression, low stability of mutant mRNA or protein could be an alternative explanation. However, this possibility is unlikely for two reasons: 1) both constructs were capable of activating a C1-Sh-GUS reporter gene in maize protoplasts: 85/87 and 87/88 activated C1-Sh-GUS at a level of 77-84% or 56% of the wild-type VP1 construct, respectively (V. Vasil, L. Rosenkrans et al., unpublished results). 2) co-expression of 87/88 as well as the double-deletion mutant 85/88 exhibited a dominant negative effect on repression of Amy-GUS by wild-type VP1 in barley aleurone, indicating presence of mutant protein in transformed cells.

The strongly positively charged, 15-amino acid-domain (RED domain), deleted in 85/87, is highly conserved (ca. 80%) among maize, barley and rice genes (Fig. 25). Between maize and barley, 11 out of 15 amino acids are identical and one amino acid constitutes a conservative substitution (R to K) also found in rice. The high degree of sequence conservation is consistent with the finding that this region is of functional importance for repression of Amy-GUS. Interestingly, deletion of the RED domain exhibited a differential effect in maize and barley aleurone. While 85/87 was incapable of inhibiting Amy-GUS in barley aleurone, it retained ca. 50% of wild-type VP1 activity in maize aleurone cells (Fig. 18). In maize, deletion of additional sequences 5' to the 85/87 deletion (construct 86/87) was necessary to eliminate repressor function (Fig. 18). This indicates that in the barley cell the RED domain is absolutely essential for proper execution of the repression mechanism, whereas in the more concordant system of the maize aleurone cell other possibly less conserved regions in the VP1 protein partially compensate in function for the RED domain.

The construct 87/88 that deletes a large but poorly conserved stretch of VP1 was incapable of repressing Amy-GUS in maize or barley cells. To further analyze this domain of VP1, four smaller deletions within this region were constructed and tested (Fig. 18). The three deletion constructs 87/92 (deleting 29 aa), 92/181 (deleting 26 aa) and 93/95 (deleting 40 aa) of which each lacks a short stretch of conserved sequence were not severely impaired in repressing Amy-GUS. In contrast, the construct 196/88 deleting 48 amino acids at the C-terminal end of the 87/88 deletion was incapable of repression in barley (no data for this construct were obtained in maize). These results allow at least two interpretations: 1) 196/88 deletes a domain essential for repression, while the sequences located between the deletion points 87 and 95 are not required for repressor function. 2) The partially conserved sequences between 87 and 95 are of redundant function. Therefore, deletion of two or more conserved blocks may be necessary to lose repression of Amy-GUS. In this interpretation, 196/88 may delete an additional important domain or, alternatively, affect proper spacing between further N-termnal and C-terminal sequences.


Z.m. MEA-SSGSSPPHSQENPPEH GGD------M-GG------AP-AEEI GGEAA-------DDF 39
H.v. MDA-SAGPPPPRHPQGSALRRGKG----------P-AVEIRHGE---------DDF 34
O.s. MDA-SAGSSAPHSHGNPGKQ-GGG-------GGGGGGRGKAP-AAEIR-GEAAR------DDV 46
A.t. MKSLHVAANAGDLAEDCGIL-GGDADDTVLMDGIDEVGREIWLDD---HGGDNNHVHGHQDDL 60
Z.m. MFAED--TF----PSLPDFPCLSSPSSSTFSSN------SSSNSSSAYTNTAGRA-G-----G 86
H.v. MFAQD--TF----PAFPDFPCLSSPSSSAADIV----------LCG 64
O.s. FFADD--TF----PLLPDFPCLSSPSSSTFSSS------SSSNSSSAFTTAAGGGCG-----G 94
A.t. IVHHDPSIFYGDLPTLPDFPCMSSSSSSSTSPAPVNAIVSSASSSSAASSSTSSAASWAILRS 123
Z.m. EPSEPASAGEGFDA---LDDIDQLLDFASLSM--PWDSE-P---------- 125
H.v. EPSEPAAAGDGMDD---LSDIDHLLDLASINDDVPWDDE-PL---------- 102
O.s. EPSEPASAADGFGE---LADIDQLLDLASLS--VPWEAEQPL---------- 135
A.t. DGEDPTPNQNQYASGNCDDSSGALQSTASMEIPLDSSQGFGCGEGGGDCIDMMETFGYMDLLD 186
86
Z.m. ---FP-GVSMMLENAMSAPPQPVGD--GMSEEKA--VPEGTT---GGEEACM-DAS--EG-EE 163
H.v. ---FP-DVGMMLEDVISEQQQQQQQHPLAGHGAGGRVASDTAGG-GGEDAFHGGGGSGSAADD 160
O.s. ---FPDDVGMMIEDAMSGQPHQADDCTGDGDTKA--VMEAAGGGDDAGDACM-E-GS-DAPDD 179
A.t. SNEFFDTSAIFSQDDDTQNPNLMDQTLERQEDQV-VVPMMENNS-GQDMQMM--NSSLEQDDD 240
85
Z.m. LPRFFMEWLTSNRENISAEDLRGIRLRRSTIEAAAARLGGGRQGTMQLLKLILTWVQNHHLQR 230
H.v. LPRFFMEWLTNIRDCISAEDLLSIRLRRSTIETTTALLGGGRQDTMQLLKLILTWVQSHHLQK 223
O.s. LPAFFMEWLTSNREYISADDLRSIRIRRSTIEAAAARLGGGRQGTMQLLKLILTWVQNHHLQK 247
A.t. LAAVFLEWLKNNKETVSAEDLRKVKIKKATIESAARRLGGGKEAMKQLLKLILEWVQTNHLQR 308
87 92 93
Z.m. KRPRDVMEE-EA-GLHVQLPSPVANPPGYEFPAGGQDMAAGGGTSWM---PHQQAFTPPAAYG 288
H.v. KRPRVGAMDQEAPPAGGQLPSPGANPS-YEFPT---ETGAAAATSWM---PY-QAFSPTASYG 278
O.s. KRPRTAIDDGAA-SSDPQLPSPGANP-GYEFPSGGQEMGSAAATSWM---PYQ-AFTPPAAYG 304
A.t. RRTTTTTTNLSY-QQSFQQDPFQNPNPNNNNLIPPSDQTCFSPSTWVPPPPQQQAFVSDPGFG 370
181 196,95
Z.m. GDAVYPSAAGQQYSFHQGPSTSSVVVNSQPFSPP---PVGDMH---GANMAWPQQYVPFPPPG 345
H.v. GEAMYPFQ--------QGCSTSSVAVSSQPFSPP--AAA-DMHA--G---AWPLQYAAFVPAG 325
O.s. GDAMYPGAAG-PFPFQQSCSKSSVVVSSQPFSPPTAAAAGDMHASGGGNMAWPQQFAPF--PV 364
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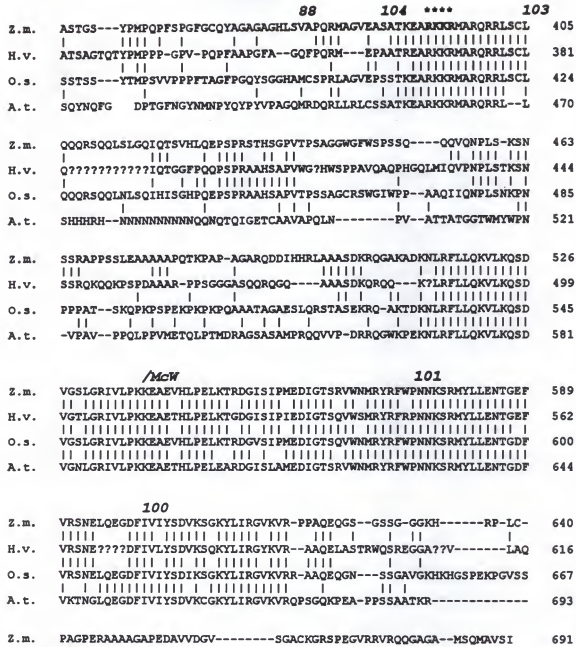


Fig. 25. Alignment of the amino acid sequences of VP1 from maize (Z.m.), HVVP1 from barley (H.v.), OSVP1 from rice (O.s.) and ABI3 from *Arabidopsis* (A.t.). Identical amino acids are shown by vertical lines. Conserved regions among the four species are boxed. Italic numbers above sequence show site of deletion points. Underlined amino acids indicate location of putative leucine zipper. Asterisks indicate putative NLS.

In summary, using single-deletion mutants, amino acid residues within the region from 222 to 374 of VP1 were found to be important for repression of Amy-GUS. Further functional analysis of sequences outside this region was conducted by taking advantage of the dominant negative effect of the 87/88 mutant on repression of Amy-GUS by wild-type VP1: In the presence of GA, co-expression of 87/88 with VP1 and Amy-GUS severely reduced repression of Amy-GUS by VP1 (Fig. 22). Domains essential for mediating the dominant negative effect of 87/88 are most likely also involved in function of the wild-type VP1 protein. 87/88 may exert a dominant negative effect for instance by competing with wild-type VP1 for binding to a component of the repression mechanism or - if VP1 functions as a dimer - by forming non-functional heterodimers with wild-type VP1. To identify domains in the 87/88 mutant that are required for expressing the dominant negative effect, double-deletion mutants between 87/88 and other deletion mutants were constructed and tested. The RED domain deleted in the 85/87 mutant was considered a putative domain because it has an important function in repression. However, the double-deletion mutant 85/88 was as effective in causing a dominant negative effect as the 87/88 single mutant, indicating that the RED domain is not essential for this effect (Fig. 23). Similar results were obtained for the domain 101/100 (Fig. 24). In contrast, the double mutants deleting 87/88 and either the C-terminal 450 bp of VP1 (87/88:McW) or the highly basic domain 103/104 (87/103) did not exhibit a dominant negative effect on Amy-GUS repression by co-expressed VP1 (Figs. 23, 24). Although it cannot be ruled out that these double mutant constructs express instable proteins, these data suggest that the C-terminus and the domain 103/104 may be required for mediating the dominant negative effect of 87/88. Deletion of these domains displayed a clearly measurable effect only if the 87/88 domain was deleted also, while the single mutants 103/104 and McW retained almost wild-type repressor function (Fig. 19). This suggests that in the single mutants other sequences can compensate in function for the deleted domains, while this is not possible in the 87/88 mutant. However, stability of the mutant proteins needs to be confirmed, especially for the 87/88:McW double mutant which deletes ca. 42% of the VP1 sequence.

In summary, deletion analysis of the VP1 protein has allowed the identification of several domains that are essential for repressor function: 1) the conserved, highly charged RED domain 85/87 and 2) the poorly conserved region 87/88. Moreover, though not essential, the domain 103/104 and the C-terminus may play a role in repression.

When comparing the repressor domains of VP1 with the domains required for transcriptional activation of the *Em* or *C1* genes, it is evident that different functions of VP1 map to different sequences in the protein (Fig. 26). Apart from the differential requirement of the acidic activation domain at the N-terminus of VP1, *α-amylase* repression and *Em* activation differed in the need for the highly positively charged domain 103/104 of VP1. While deletion of 103/104 reduced Em-GUS activation by 98% in maize protoplasts (L. Rosenkrans et al., unpublished results), it did not severely affect repression of Amy-GUS. Likewise, *α-amylase* repression and *C1* activation displayed a differential requirement for the C-terminal part of VP1. Deletion of the C-terminal ca. 150 amino acids of VP1 entirely eliminated activation of C1-Sh-GUS (L. Rosenkrans et al., unpublished results). In contrast, this domain was not found essential for repression of Amy-GUS.

Similarly, different domains of VP1 are essential for activation of *C1* and activation of *Em*. Overall, sequences required for induction of anthocyanin biosynthesis map to the highly conserved C-terminal end of VP1, while sequences essential for activating *Em* are located in the central part of VP1 (85/87, 87/88 and 103/104). These findings are consistent with the phenotype of mutant alleles in maize and *Arabidopsis*. The maize *vp1-McW* allele truncating the C-terminal ca. 150 amino acids of VP1 produces seeds exhibiting nearly normal developmental arrest but lack of anthocyanin accumulation, indicating that the C-terminal part of VP1 is not essential for preventing vivipary but is essential for anthocyanin production (McCarty et al., 1989b). In *Arabidopsis*, two *abi-3* alleles that produce a viviparous phenotype have been sequenced: *abi3-6* contains an internal deletion of ca. 750 bp between positions 1,073 and 1,944 (Nambara et al., 1994), thus deleting the domains corresponding to 196/88 and 103/104. The mutation in *abi3-3* induces a premature stop codon at Gln^{417} (Giraudat et al., 1992), thus deleting

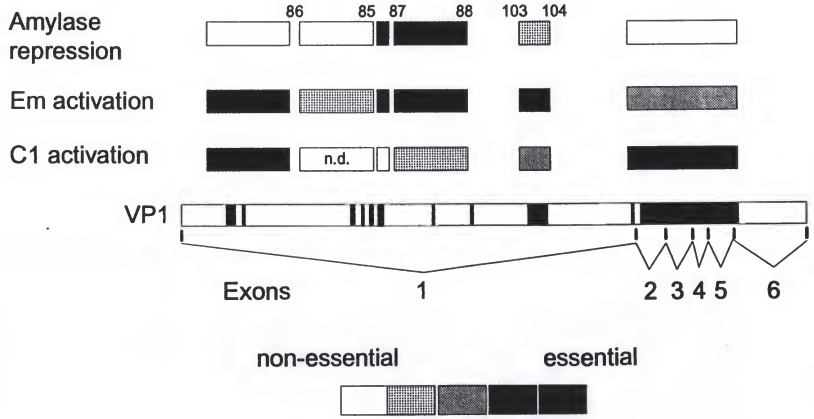


Fig. 26. Summary on domains of VP1 involved in repression of α-amylase genes, activation of *C1* and activation of *Em*.

the domain 103/104 and the C-terminus.

VP1 shows no significant sequence homology to other proteins. Therefore, the function of domains other than the acidic activation sequence is thus far unknown. It was suggested that the region from amino acid 208 to 235 of the rice VP1 may form a leucine zipper-like structure (Hattori et al., 1994). In this region, Leu or Ile residues are located at every seventh residue (5 repeats) with an exception for the fourth position (see Fig. 25). However, no severe loss-of-function phenotype has been observed when this domain was deleted in the 86/85 construct. 86/85 effected full repression of Amy-GUS and ca. 50% of wild-type activity with respect to activation of Em-GUS. These data do not support an important role of this domain in protein-protein interactions.

Based on the evidence that VP1 transcriptionally activates *C1* and *Em* (McCarty et al., 1991; Hattori et al., 1992), nuclear targeting is likely to be a requirement for VP1 function. A 100% conserved putative nuclear localization sequence (NLS), RKKR, exists in the domain from amino acid 392 to 395 of VP1 (see Fig. 25), as mentioned by Giraudat et al. (1992). Consistent with these views, deletion of this putative NLS (construct 103/104) fully eliminated activation of Em-GUS (L. Rosenkrans et al., unpublished results). However, 103/104 retained capacity to activate C1-Sh-GUS (31% of wild-type VP1), suggesting that the mutant protein is targeted to the nucleus – though possibly with reduced efficiency. Assuming that RKKR is a functional NLS, these data indicate that VP1 contains two or more NLSs with at least partially redundant function, a feature not uncommon among nuclear proteins (Raikhel, 1992). The differential effects of 103/104 on activation of *Em* and *C1* may reflect different threshold levels of VP1 protein required for these activator functions. Hence, the extent of nuclear localization of 103/104 may be sufficient for partial activation of *C1* but not for activation of *Em*. Alternatively, 103/104 may serve an additional function in activation of *Em*. A dual role of a domain in both nuclear targeting and DNA binding was reported for the regulatory protein O2 (Varagona et al., 1994).

With respect to repression of Amy-GUS by VP1, it is unknown whether nuclear localization of VP1 is required for function. The construct 103/104 was not affected in repression

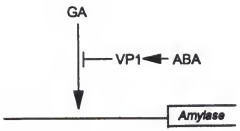
of Amy-GUS. This may indicate that nuclear targeting of VP1 is not required or that other domains with redundant function may compensate in function for the deletion in 103/104. Interestingly, the double-deletion mutant 87/103 lost the ability to exert a dominant negative effect on Amy-GUS repression by VP1. A failure of the double mutant protein to be targeted to the nucleus would be consistent with the observed loss of function.

Apart from the activation domain, the biochemical functions of sequences in the VP1 protein remain unclear. However, the deletion analysis demonstrated very clearly that different domains are involved in the different functions of VP1, thus underlining the multifunctional nature of this transcription factor.

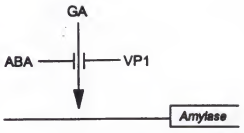
Interactions Between VP1 and Plant Hormones

One can envision at least three models of how VP1 might function in repressing α-amylase genes: 1) VP1 might mediate ABA antagonism of GA signalling during seed development. ABA is known to antagonize GA-action in the regulation of α-amylase genes in germinating cereal seeds (Jacobsen and Chandler, 1987). Because VP1 is required for ABA-induced gene expression associated with seed maturation (McCarty et al., 1991), it might also be essential in ABA-mediated repression of α-amylase genes (Fig. 27A). Consequently, the *vp1* mutant might allow de-repression of α-amylase genes by failing to respond to ABA present in the developing seed. 2) VP1 might specifically inhibit the GA-response pathway independently of ABA (Fig. 27B). 3) VP1 might repress α-amylase genes via a pathway that functions independently of both GA and ABA (Fig. 27C).

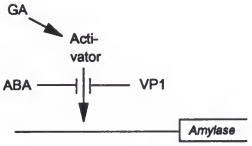
The results presented in this work do not support the first model. ABA was effective in blocking Amy-GUS expression in *vp1* mutant aleurone cells (Fig. 6), indicating that ABA action in this instance does not depend on the presence of VP1. In combination, VP1 and ABA effects were roughly additive. This stands in contrast to evidence showing that VP1 is required for ABA-induced expression of the maize *Em* gene (McCarty et al., 1991). Thus, there appear to be at



A. VP1 may be required for ABA function



B. VP1 may block GA response pathway independently of ABA



C. VP1 may repress amylase genes independently of hormones

Fig. 27. Alternative models for VP1 function as described in the text

least two modes of ABA action in the maize seed, a VP1-dependent pathway and a VP1-independent pathway. Multiple ABA transduction pathways are also indicated by interactions between ABA-insensitive mutants of *Arabidopsis* (Finkelstein and Somerville, 1990; Finkelstein, 1994). This suggests that ABA modulates the activity of diverse regulatory cascades in the seed.

The second scenario in which VP1 could specifically block GA signal transduction is supported by the evidence that over-expression of VP1 in aleurone of imbibed barley half seeds severely reduced GA-induction of Amy-GUS without affecting the basal activity of the α-amylase promoter (Fig. 11). This suggests that expression of VP1 in the developing seed may be, at least in part, responsible for the observed GA-insensitivity of cereal and maize α-amylase genes prior to seed maturity (Nicholls, 1979; Comford et al., 1986; Garcia-Maya et al., 1990; Oishi and Bewley, 1990). VP1 displayed full repressing activity in *slender* (*sln*) mutant barley seeds (Fig. 12) which are constitutive in GA response of the aleurone (Chandler, 1988; Lanahan and Ho, 1988), suggesting that VP1 functions at a point downstream of the *Sln* gene product.

With respect to the maize seed, the data do not rule out the possibility that VP1 acts independently of GA as a developmental repressor of α-amylase genes. Although we have shown that Amy-GUS is GA-inducible in *vp1* mutant aleurones early in development (Table 1), it is not clear that the high constitutive activities found later in development are entirely attributable to changes in GA concentration. In contrast to the situation of Himalaya barley seed and other cereal grains, studies of α-amylase regulation in normal and GA-deficient (*d5* mutant) genotypes of maize indicate that α-amylase induction in germinating maize seeds is largely independent of GA (Harvey and Oaks, 1974). Consistent with these studies, it was found in the present work that during germination Amy-GUS is constitutively active in the GA_1-deficient *d1* mutant of maize. Because Amy-GUS was fully VP1-repressible in aleurones of developing *vp1* mutant, germinating wild-type and germinating *d1*-mutant seeds of maize, it is suggested that VP1-mediated repression is not necessarily restricted to, nor solely defined by, inhibition of the GA response. Though the significance of GA in the expression of α-amylase genes needs to be

investigated further, it is likely that GA, ABA and VP1 are three among several factors that regulate the activity of constituents required for expression of α-amylase genes in the maize seed (Fig. 27C).

Expression experiments in developing maize seeds have shown that VP1 and ABA are likely to act independently in repressing Amy-GUS. Further evidence regarding the relative positions of VP1 and ABA in the regulatory network allowed the characterization of the dominant negative effect caused by the deletion mutant 87/88. Co-expression of 87/88 with wild-type VP1 and Amy-GUS has shown that 87/88 inhibits repression of Amy-GUS by VP1 ("dominant negative effect", Fig. 22). This suggests that the observed 87/88-mediated activation of Amy-GUS in the absence of GA (Fig. 20) may be caused by the presence of residual amounts of endogenous barley VP1 homolog in the wild-type aleurones. Because ABA was incapable of inhibiting the 87/88-mediated activation of Amy-GUS (Fig. 21), it is suggestive that VP1 functions either downstream of ABA or via a different signalling pathway than ABA. It therefore will be interesting to map the *cis*-elements in α-amylase promoters that are responsible for VP1 and ABA action.

The Role of the Embryo

Although the *vp1* mutant phenotype was cell autonomous within the aleurone of *vp1-m2* seeds (Fig. 5a,b), de-repression of α-amylase genes was not fully independent of the physiological state of the embryo: 1) in *vp1-m2* seeds, precocious hydrolization of endosperm reserves in sectors underlying *vp1* mutant aleurone was predominantly observed in seeds carrying a viviparous embryo. 2) Amy-GUS was de-repressed in *vp1* mutant aleurone of concordant *vp1* mutant seeds but not in *vp1* mutant aleurone of non-concordant seeds exhibiting a wild-type embryo (Fig. 15). These observations suggest that a viviparous embryo facilitates expression of α-amylase genes in *vp1* mutant aleurone. However, the finding that Amy-GUS is highly induced in *vp1* mutant aleurone of germless seeds (Fig. 16) indicates that a viviparous

embryo per se is not required for de-repression of Amy-GUS in *vp1* mutant aleurone. Instead, it rather appears to be the lack of a normal embryo that facilitates expression of Amy-GUS, suggesting that a wild-type embryo contributes a diffusable signal with inhibitory effect on α-amylase gene expression in the aleurone. Experimental evidence suggests that developing embryos are the major source of ABA present in the maturing seed (King, 1979; Jones and Brenner, 1987). Because Amy-GUS remains sensitive to inhibition by ABA in *vp1* mutant aleurone, ABA produced by the wild-type embryo may be responsible for the observed repression of Amy-GUS in *vp1* mutant aleurone of non-concordant seeds. This is consistent with the finding that Amy-GUS was de-repressed to a similar extent in developing aleurones of the ABA-deficient mutant *vp5* as in aleurones of the germless mutant (data not shown).

In concordant *vp1* mutant seeds, ABA concentrations are equal to, or only ca. two-fold lower than, those present in wild-type seeds (Neill et al., 1986, 1987; Paiva and Kriz, 1994). This suggests that the viviparous embryo also contributes an inductive signal (e.g. GA) that counteracts the effect of ABA and therefore uncovers de-repression of α-amylase genes in *vp1* mutant aleurone. However, the observed strong expression of Amy-GUS in *vp1* mutant aleurone of germless seeds clearly shows that GA production by the embryo is not required for α-amylase expression in maize aleurone. Supported by the evidence that the endosperm of mature cereal seeds is not a source of GA (Jacobsen and Chandler, 1987), these data suggest that Amy-GUS expression is largely independent of GA. Moreover, this interpretation is consistent with other studies (Harvey and Oaks, 1974) and findings in this work indicating that in mature seeds of GA-deficient mutants of maize α-amylase genes are expressed at high levels.

Complete repression of Amy-GUS in aleurones of developing seeds was observed only if the embryo as well as the endosperm were of wild-type genetic constitution (Fig. 16). Lack of either a normal, arrested embryo or VP1 expression in the aleurone lead to partial de-repression of Amy-GUS in aleurone cells. This indicates that neither factor -- expression of VP1 in the aleurone cells or the presence of a normal embryo -- is sufficient for total inhibition of α-amylase genes.

VP1 Integrates the Control of Seed Maturation and Germination Programs

It has been shown in this work that VP1 participates in the regulation of two developmental pathways in the developing maize seed. As a transcriptional activator it is required for activation of maturation-specific genes (McCarty et al., 1991) and as a repressor it prevents precocious induction of the normally germination-specific α-amylase genes (data presented herin). Hence, expression of VP1 specifically during seed development appears to be involved in ensuring proper ordering of maturation and germination programs. Physically combining activation and repression function in one protein appears to provide one mechanism for directly integrating control of mutually exclusive developmental pathways in the plant embryo. The importance of a tight control of maturation and germination programs for seed survival is evident in the phenotype of *vp1-m2* seeds.

Premature induction of postgerminative development was also reported for the *lec1* (*leafy cotyledon 1*) mutant of *Arabidopsis*. In this ABA-sensitive, viviparous mutant, developing embryos expressed isocitrate lyase genes and a gene encoding a lipid transfer protein at levels that are normally characteristic of seedlings (West et al., 1994). Double mutant analysis suggested that the putative *Arabidopsis* VP1 homolog, ABI3, and LEC1 function in different pathways (Meinke et al., 1994). Hence, it appears that multiple mechanisms have evolved in flowering plants to prevent precocious induction of normally germination-specific genes in the developing embryo.

Thus far, the evidence that VP1 inhibits germination-specific genes is limited to hydrolase genes in aleurone cells. It is unknown to what extent this repressor activity of VP1 is also involved in preventing precocious germination of the embryo. Further insight into the inhibitory role of VP1 during seed development may be provided by stable transformation of *vp1* mutant plants with VP1-derivatives that are mutated specifically in the activator or repressor function.

Cloning of the *Vp1* related genes from barley (M. Stoll and D.R. McCarty, unpublished results), rice (Hattori et al., 1994), *Arabidopsis* (Giraudat et al., 1992) and tobacco (Phillips and

Conrad, 1994) indicates that the *Vp1* gene is conserved among flowering plants. Loss of ABI3 function in *Arabidopsis* causes a similar viviparous phenotype as the *vp1* mutation in maize (Nambara et al., 1992). The functions of ABI3 and VP1, however, diverge in so far that ABI3 is required for seed dormancy in *Arabidopsis* while VP1 does not impose seed dormancy in maize. Because ABI3 mRNA is stored in the dry seed (Parcy et al., 1994), whereas VP1 transcript and protein are non-detectable in the mature seed (McCarty et al., 1989; Carson, 1992), one can speculate that dormancy in *Arabidopsis* may reflect an extended timing of ABI3 expression after seed maturity rather than a functional difference in the proteins. This view is supported by the results showing that over-expression of VP1 in aleurone of germinating maize seeds was effective in repressing Amy-GUS. A role of VP1 in maintaining seed dormancy is also consistent with the finding that dormancy in barley is correlated with a reduced GA-inducibility of α-amylase genes in the aleurone (Schuurink et al., 1992; Skadsen, 1993). Hence, it is suggested that VP1 plays a role in integrating the control of seed maturation, dormancy and germination programs.

SUMMARY AND CONCLUSIONS

The Viviparous-1 (VP1) transcriptional activator of maize is required for abscisic acid-induction of maturation-specific genes late in seed development leading to acquisition of desiccation tolerance and arrest in embryo growth (McCarty et al., 1991). The presented research extends these findings by showing that VP1, in addition to its transcriptional activation function, inhibits precocious induction of the germination-specific α-amylase genes in aleurone cells of the developing seed. Functional analysis of deletion-derivatives of VP1 in a transient gene expression system indicated that VP1 has a discrete repressor function that is separable from its transcriptional activation function. It is therefore suggested that physically combining activator and repressor functions in one protein provides one mechanism for directly integrating control of the mutually exclusive developmental pathways, seed maturation and seed germination, in the plant embryo.

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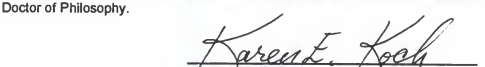

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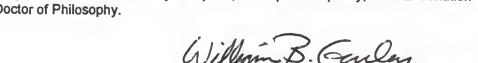

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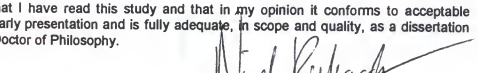

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